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January-April, 1949 (inclusive)

VOLUME 70

NEW YORK

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SCIENTIFIC PROCEEDINGS, VOLUME 70

Press of
THOMAS J. GRIFFITHS SONS, INC.
Utica, N. Y.

Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 70 JANUARY, 1949 No. 1

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16805

Wallingford Poliomyelitis Virus: Another Strain of the Lansing Type, Infective in Rodents.*

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Since the original isolation by Armstrong[1] of poliomyelitis virus capable of infecting rodents, 4 other strains immunologically related to the Lansing virus and also infective in rodents have been established. One of these is the M.E.F.1. strain, isolated from the central nervous system (C.N.S.) of a fatal case in the British Middle East Forces in 1942.[2,3] Two others isolated by investigators at Yale are the S.K. and Phillips strains. The first was obtained from feces of a non-paralytic patient in New Haven in 1937[4,5] and the second was obtained from the C.N.S. of a fatal case in Egypt in 1943.[5] A fourth strain (W.W.)[6] was isolated from the blood-stream of a patient in New York in 1946, although such blood-stream isolations of poliomyelitis virus are very rare events.

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

1 Armstrong, C., *Pub. Health Rep.*, 1939, **54**, 1719.

2 Van Rooyen, C. E., and Morgan, A. D., *Edinburgh Med. J.*, 1943, **50**, 705.

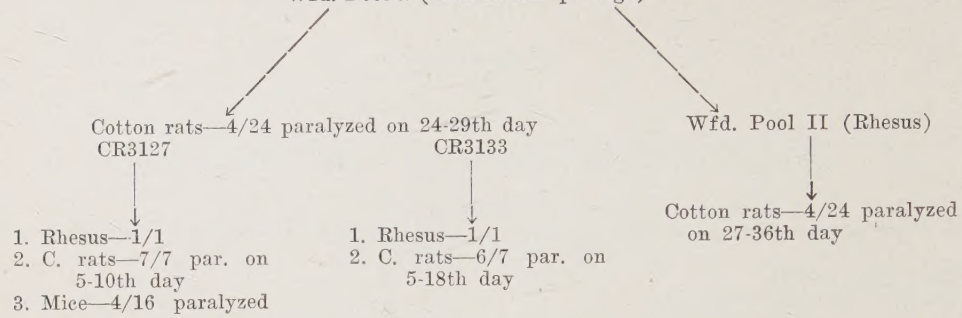
3 Schlesinger, R. W., Morgan, I. M., and Olitsky, P. K., *Science*, 1943, **98**, 452.

4 Trask, J. D., Vignec, A. J., and Paul, J. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 147.

5 Melnick, J. L., and Ward, R., *Fed. Proc.*, 1948, **7**, 308.

6 Koprowski, H., Norton, T. W., and McDermott, W., *Pub. Health Rep.*, 1947, **62**, 1467.

CHART 1.
Passage of 10% Wfd. Virus Suspension in Rodents.
Wfd. Pool I (16th Rhesus passage).



In the course of investigations of strain differences, in which it was clearly shown that three distinct types of poliomyelitis viruses can be identified, the suspicion arose that another strain used in this laboratory—the Wallingford (Wfd.)—might be immunologically related to Lansing virus. This strain was found to be unrelated to the Brunhilde virus, as will be shown, and since the Brunhilde strain is immunologically distinct from the Lansing, it was decided to compare the Wfd. with the Lansing strain. The Wfd. strain was isolated in California in 1934 by Trask and Paul and was soon described as a strain of unusual infectivity by intracutaneous inoculation in monkeys.[7] It was obtained in this laboratory in 1938 through the courtesy of the late Dr. James Trask, and was used in experiments to utilize its apparently high infectivity by the intracutaneous route until 1940. It was then in its sixteenth rhesus passage, having been maintained as an aqueous 20% suspension, stored in a dry-ice chest, since it was obtained in the form of glycerolated cord, in the eleventh rhesus passage, from Dr. Trask.

A pool of cords from 2 rhesus monkeys, both representing the sixteenth rhesus passage (Wfd. Pool I), and a second pool (Wfd. II) derived from 3 monkey cords infected with virus from Wfd. Pool I, were used for the rodent passage experiments. The Pool I suspension had been stored in a dry-ice chest from 1940 to 1948. Chart I summarizes the results of these rodent passages and the reverse passage back to rhesus monkeys.

It will be noted that in the first passage to cotton rats of Pool I and Pool II, not a single incubation period was less than 24 days, among 8 cotton rats paralysed. In contrast, cotton rats inoculated with material from 2 cotton rats, paralysed in the first rodent passage, had much shorter incubation periods (11 of 14 between 5 and 10 days). The cords of these 2 cotton rats each infected the single rhesus monkey inoculated therewith, and the one cotton rat cord inoculated intracerebrally in mice paralysed 4 of 16. The infected rhesus monkeys showed typical paralytic poliomyelitis and, as was the case with the cotton rats, exhibited typical poliomyelitis lesions in the brain and spinal cord.

Immunological relationship to Lansing virus. The Wfd. virus was shown to be immunologically closely related to the Lansing virus and unrelated to the Brunhilde virus (Table I). Four rhesus monkeys immunized intramuscularly with the active Brunhilde virus, and shown to be immune to intracerebral challenge with 10,000 PD 50 of the Brunhilde virus, according to the method previously described,[8] promptly succumbed to intracerebral challenge with 0.8 cc of 10^{-1} cord suspension of Wfd. Pool I. All 4 had severe paralytic poliomyelitis, confirmed by histopathological examination of the central nervous system.

Four rhesus monkeys similarly immunized with, and immune to, Lansing virus were also challenged in the same fashion with virus of Wfd. Pool I. Ten normal controls were simi-

[7] Trask, J. D., and Paul, J. R., *J. Bact.*, 1936, **31**, 527.

[8] Morgan, I. M., *Am. J. Hyg.*, in press.

TABLE I.
Immunological Relationship of Wfd. Poliomyelitis Virus to Lansing and Brunhilde Strains.

Immunizing virus	Inoculated intracerebrally with Wfd. virus: Convalescent rhesus monkeys	Inoculated intracerebrally with Wfd. virus: Vaccinated monkeys shown to resist immunizing virus
Brunhilde	4/4	4/4
Lansing	0/3	1/4
Normal controls	10/10	

larly challenged. All 10 controls succumbed to poliomyelitis infection, whereas only one of the 4 Lansing-immune monkeys was paralysed. Since these immunized monkeys had, because of the summer vacation, been challenged 2½ months after their last experience with Lansing virus, it was thought that a drop in antibody titer might have accounted for the failure of the one monkey to resist. Accordingly, his serum was titrated against $10^{-2.5}$ ($=10$ LD_{50}) Lansing virus in mice, and was found to have a neutralizing titer of 1 in 300. Since it is known that at this level only about 50% of monkeys are protected, and that the level of serum antibody associated with practically complete intracerebral immunity is of the order of 1 in 1,000,[9] it is likely that complete immunity of the 4 Lansing-immune monkeys to Wfd. virus would have resulted if the animals had been challenged no longer than one month after the course of immunization. A quite comparable result was obtained by challenge of monkeys with Wfd. virus several months after recovery from attacks of poliomyelitis with Lansing and Brunhilde viruses, respectively (Table I).

Discussion. It is interesting that the Wfd. virus, along with another virus (McC.) isolated in the same epidemic from nasal washings of a non-paralytic patient,[10] and the Aycock-1920 strain, were all considered to be related to the S. K. virus, as determined by neutralization tests.[11] Since the S.K. virus is immunologically related to the Lansing virus, and infective in rodents, further evidence regarding the affinities of the McC. and Aycock-1920 strains would be of interest. Aside from these 2 viruses, it appears that at least 6 other established strains are either definitely or very likely closely related to the Lansing virus by immunological test. Of these, 5 have thus far been passaged in rodents.

On the basis of intracerebral neutralization tests in mice, in which it was found that convalescent serum of monkeys infected with M.E.F.2. virus (Egypt, 1942) neutralized Lansing virus and M.E.F.2. virus, Schlesinger, Morgan and Olitsky concluded that M.E.F.2. virus was serologically of the Lansing type.[3] Our own cross-immunity experiments, however, indicate that rhesus monkeys immunized with, and immune to, Lansing virus may still be infected with M.E.F.2. virus. On the contrary, animals immunized with, and immune to, Brunhilde virus (known to be unrelated to Lansing serologically and immunologically) are completely resistant to M.E.F.2. virus.[12] This virus, therefore, cannot be considered to be primarily of the Lansing type. As shown by Schlesinger, *et al.*, it is not infective in cotton rats. Since a third strain (M.E.F.6.) studied from the Egyptian outbreak in 1942 in the Middle East Forces by the same authors, was shown by them to be serologically unrelated to the Lansing virus, it appears not unlikely from their results and from ours that 3 distinct strains produced fatal illness in that outbreak. The first, M.E.F.1., is of the Lansing type, and is infective in rodents. The second, M.E.F.2., although serologically related to the Lansing virus, at least to some extent, is more closely related to a known non-Lansing strain, the Brunhilde, by cross-immunity experi-

9 Morgan, I. M., to be published.

10 Paul, J. R., Trask, J. D., and Webster, L. T., *J. Exp. Med.*, 1935, **62**, 245.

11 Trask, J. D., Paul, J. R., and Vignec, A. J., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 241.

12 Bodian, D., Morgan, I. M., and Howe, H. A., *Am. J. Hyg.*, in press.

ments. The third, M.E.F.6., is apparently unrelated serologically to the Lansing strain, but more details of its affinities are needed before it can be stated that it is entirely unrelated to the Lansing.

The 9 strains either demonstrated or thought to be of the Lansing type, are listed below:

Lansing	—1938—Mich.[1]	Passaged in rodents
M.E.F.1	—1942—Egypt[3]	
Y-S.K.	—1937—New Haven[5,11]	
Phillips	—1943—Egypt[5]	
Wfd.	—1934—Los Angeles[7]	
W.W.	—1946—N. Y.[6]	
M.V.	—1909-1914—N. Y.	Not passaged in rodents
McC.	—1934—Los Angeles[10](?)	
Aycock	—1920—Vt.[11](?)	

Three of the above strains (M.E.F.1., Wfd., M.V.) have been shown in this laboratory to be not only closely related to the Lansing, but unrelated to the Brunhilde virus by tests of vaccination-immunity. Seven other strains isolated in this country from 1939 to 1946, have been shown to be related to the Brunhilde virus by the same method.[12] It seems evident, therefore, that any virus shown either to be unrelated to strains of the Brunhilde type, or related to those of the Lansing type should be investigated with respect to possible rodent pathogenicity. Such tests should be done in cotton rats rather than in mice, because of the known greater susceptibility of the former to the Lansing virus,[13] and moreover a large number of cotton rats should be inoculated. Observations of inoculated cotton rats should be continued for at least 2 months, since, as shown by Armstrong,[1] by Schlesinger, *et al.*,[3] and by our results, incubation periods may be of long duration.

It is interesting that of the six strains known to be closely related immunologically to the Lansing strain, five have been passaged in rodents. This fact leads to the strong suspicion that rodent pathogenicity is closely linked to the specific antigenic structure of this group of viruses. At first sight the MV strain appears to be an exception, but since it has had a long series of monkey passages since its isolation some 40 years ago, it may be that it originally possessed the property of rodent infectivity, but lost it because of numerous monkey transfers. Another question which may be raised is whether strains which are known not to be related immunologically to the Brunhilde virus are likely to be related to the Lansing strain. It was this supposition which led to the successful passage of Wallingford virus in rodents. Following the same suggestion, an attempt was made to passage the Leon strain in cotton rats because of our finding that it was not related to the Brunhilde virus, by tests of vaccination-immunity. This strain was isolated in Los Angeles in 1937, by Dr. John F. Kessel, who kindly supplied our sample. We have failed to passage the Leon strain in cotton rats, and moreover have shown that it is immunologically unrelated to both the Brunhilde and Lansing viruses.[12] The Leon strain is therefore representative of a third distinct immunological group of poliomyelitis viruses. It will be of interest to know whether other representatives of this group will be identified and whether they also lack the property of rodent infectivity.

It is of interest that when known Lansing-like viruses are examined as a group, they appear to be widely distributed geographically, and can be isolated from both paralytic and non-paralytic cases. Since they further occur in nasopharynx and feces, as well as in the C.N.S., and since Lansing antibodies are widely distributed in human serum, there seems to be no reason to doubt at the present time, that these viruses are important in the epidemiology of poliomyelitis. It remains to be seen whether they are of less importance in this regard than viruses of the non-Lansing type.

Summary. The Wallingford virus, isolated from the nervous system of a fatal poliomyelitis case in California in 1934, has been successfully passaged in cotton rats and in mice. Material from each of 2 paralysed cotton rats produced typical poliomyelitis in one rhesus monkey. The virus has been tested for immunological relationships by inoculation in monkeys vaccinated with, and shown to be immune to, the Lansing and the Brunhilde viruses, respectively. These two viruses are

[13] Bodian, D., Morgan, I. M., and Schwerdt, C. E., to be published.

representatives of 2 distinct types of poliomyelitis virus. The Wallingford virus is indistinguishable from the Lansing virus by the vaccination-immunity test, and unrelated to the Brunhilde virus by the same test.

A relationship between the property of rodent infectivity and the immunological specificity of the Lansing group of poliomyelitis viruses is suggested, in view of evidence for the existence of three distinct immunological groups of poliomyelitis viruses, only one of which has thus far been shown to be infective in rodents.

16806

Transmission of the Hamster-Adapted Newcastle Virus to Swiss Albino Mice.

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Following the adaptation of Newcastle virus, California Strain No. 11914, to the Syrian hamster,[1] attempts to transmit the modified virus of the 8th hamster passage to Swiss albino mice were successful through the fourth serial subinoculation only.[2] Brandly, *et al.*[3] working with egg propagated Newcastle virus reported similar results.

Further attempts to establish the infection in Swiss albino mice through the injection of hamster-adapted Newcastle virus of later hamster passages were likewise unsuccessful beyond several subinoculations.

Concentrated virus of the 203rd hamster passage was prepared from 8 infected hamster brains. The brain tissue was ground with alundum and diluted to a 10% suspension with physiological saline solution. Eighteen cc of the suspension were centrifugated under aseptic conditions at 50,000 RPM for 2 hours in a Spinco ultra-centrifuge. The supernate was discarded and the sediment was resuspended in 3 cc of physiological saline solution. Approximately .05 cc of the concentrated virus suspension was injected intracerebrally into each of 12 Swiss albino mice CFW.*

The use of this concentrated virus of the 203rd passage for the initial mouse inoculation established an infection in which slightly more than 10% of the injected mice of the first 8 passages showed symptoms of central nervous system involvement. From the 9th through the 20th passages more than 60% of the injected mice showed the characteristic symptoms. Table I shows the results of these 20 serial passages.

In mice responding to the virus injection irritability and malaise appeared in 2 to 9 days. In most cases these symptoms were followed by paralysis, evidenced first in the forelegs and followed in some cases by complete prostration within several hours. In other cases complete paralysis did not appear for as long as 24 hours after the forelegs became affected. Rhythmical jerking of groups of muscles or the entire body of paralyzed mice was frequently noted, accompanied by labored respirations. Death occurred within a few hours after the animals became moribund.

Brain material for passage was chosen from mice sacrificed when nervous symptoms developed. The virus-bearing brain material was ground with alundum and diluted to 10% suspension with physiological saline solution.

1 Reagan, R. L., Lillie, M. G., Poelma, L. J., and Brueckner, A. L., *Am. J. Vet. Res.*, 1947, **8**, 136.

2 Reagan, R. L., Lillie, M. G., Poelma, L. J., and Brueckner, A. L., *Am. J. Vet. Res.*, 1947, **8**, 427.

3 Brandly, C. A., Moses, H. E., Jungherr, E. L., and Jones, E. E., *Am. J. Vet. Res.*, 1946, **7**, 289.

* The Webster strain of Swiss albino mice from Carworth Farms, Rockland Co., N. Y., weighing between 10 and 12 g were used for all passages.

TABLE I.
Mouse Passage of a Strain of Hamster-Adapted Newcastle Virus.

Passage No.	No. animals inoculated*	Animals paralyzed moribund or dead		No. days after inoculation paralysis occurred
		No.	%	
1	12	2	17	4-5
2	10	1	10	6
3	12	2	17	5-6
4	16	1	6	6
5	18	2	11	3-5
6	10	3	30	3-4-5
7	17	2	12	2-3
8	18	2	11	4-6
9	14	2	14	4-5
10	18	4	22	2-3-4-7
11	20	4	20	2-3-6-7
12	12	4	33	4-6-7-9
13	10	7	70	2-3-4
14	25	16	64	2-3-4
15	22	13	59	2-3
16	10	10	100	1
17	8	8	100	1-2
18	18	16	88	1-2-3
19	28	24	86	2-3-4-5
20	20	17	85	2-3-4-5

* Intracerebrally.

TABLE II.
Neutralization Test Results.

	Virus dilutions		
	10^{-1}	10^{-2}	10^{-3}
Specific chicken antiserum	0/4	0/4	0/5
Normal chicken serum	4/4	4/4	1/4
Virus titration, 15th mouse passage	7/7	4/4	2/4

Numerator of fraction denotes the number of deaths.
Denominator of fraction denotes number of mice inoculated.

Amounts of .03 to .05 cc of this suspension were injected intracerebrally throughout the 20 passages.

A virus neutralization test was conducted using brain suspension of the 15th mouse passage with specific Newcastle virus immune and normal chicken sera and with mice as the test animals. Specific immune serum completely neutralized the mouse brain virus whereas normal chicken serum had no effect. The virus titrated 10^{-3} according to the Reed-Muench calculation.[4] Details are given in Table II.

Infected mouse brain suspension of the 8th passage prepared as previously described was injected intracerebrally in 4 young Syrian hamsters. All hamsters showed symptoms characteristically seen in animals succumbing to similar injections of hamster adapted virus. The same results were observed in hamsters injected with mouse brain of the 18th mouse passage.

Summary. The hamster-adapted Newcastle virus (California strain Number 11914) of the 203rd passage has been successfully transmitted to Swiss albino mice and carried through 20 serial passages in this species by intracerebral inoculation. The virus produced symptoms of irritability and malaise usually followed by paralysis, and often accompanied by a characteristic nervous jerking with labored breathing. Mice showing typical symptoms of central nervous system involvement did not recover. Positive Newcastle chicken serum neutralized the virus from the 15th mouse passage while normal chicken serum failed to neutralize the virus. The virus of the 15th mouse passage titred 10^{-3} in mice by intracerebral inoculation. The mouse-adapted virus proved pathogenic for Syrian hamsters upon intracerebral injection.

The authors wish to express appreciation to Miss Dorothy M. Schenck for technical assistance.

[4] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

16807

Histochemical Specificity of Phosphatases.*

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The question of the unity or plurality of phosphatases (Ph) has been a moot point for over 20 years, with numerous champions on both sides. There can be no doubt that alkaline and acid Ph are distinctly different enzymes; also the individuality of adenosinetriPh of muscle,[1] of pyroPh[2] and of hexosediPh[3] appears to be firmly established. However, a number of workers maintain that, besides these generally recognized differences, Phs differ among themselves in 3 more respects: 1 substrate specificity; 2, organ specificity and 3, specific activation and inhibition effects. The latter 2 groups often overlap.

As far as substrates specificity is concerned, Forrai[4] thinks that there is a specific sucrosePh and other sugarPhs; according to King,[5] lecithin is dephosphorylated by an enzyme not identical with the nonspecific Ph; Roche and Latreille[6] maintain that the kidney contains, besides glyceroPh, a phenylPh; Reis[7,8] claims the existence of a 5-nucleotidase; Bowers and coworkers[9] believe that aminoethylphosphate is hydrolyzed by a special enzyme; Waldschmidt-Leitz and Koehler,[10] Ichihara,[11] and Bredereck and Geyer[12] maintain that there is a special "phosphamidase" hydrolyzing the P-N bond.

There are data available to the effect that alkaline Phs of different organs may be actually different enzymes as shown by their slightly different resistance to inhibitors or by different pH optima. Belfanti and coworkers,[13] and Hommerberg[14] believe that bone Ph is different from renal or hepatic Ph. Bodansky[15] thinks that intestinal Ph can be distinguished from bone or renal enzyme. Masayama and Shuto[16] found a Ph in hepatomas, different from that of the normal liver.

Cloetens[17,18] distinguishes two alkaline Phs on the basis of their different degrees of activation by Mg. Drill, Annegers and Ivy[19] find that in jaundice a Ph appears in the plasma, different from the normal enzyme in respect to inactivation by cyanide.

Whether the differences reported should be considered as indications of the existence of several truly different enzymes, or as results of the admixture of various activators and inhibitors or of differences in technic cannot be decided on the basis of data available. The question is: Can histochemical technic be

* This work has been done under grants from the Douglas Smith Foundation for Medical Research of The University of Chicago, and from the Pathology Study Section of the U. S. Public Health Service.

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6 Roche, J., and Latreille, M., *Enzymologia*, 1937, **3**, 75.

7 Reis, J., *Enzymologia*, 1937-38, **2**, 110.

8 Reis, J., *Enzymologia*, 1938, **5**, 251.

9 Bowers, R. V., Outhouse, E. L., and Forbes, J. C., *J. Biol. Chem.*, 1940, **132**, 675.

10 Waldschmidt-Leitz, E., and Koehler, F., *Biochem. Z.*, 1933, **258**, 360.

11 Ichihara, M., *J. Biochem.* (Japan), 1933, **18**, 87.

12 Bredereck, H., and Geyer, E., *Z. physiol. Chem.*, 1938, **254**, 223.

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14 Hommerberg, C., *Z. physiol. Chem.*, 1929, **185**, 123.

15 Bodansky, O., *J. Biol. Chem.*, 1937, **118**, 341.

16 Masayama, T., and Shuto, M., *Gann*, 1940, **34**, 176.

17 Cloetens, R., *Enzymologia*, 1939, **6**, 46.

18 Cloetens, R., *Enzymologia*, 1939, **7**, 157.

19 Drill, V. A., Annegers, J. H., and Ivy, A. C., *J. Biol. Chem.*, 1944, **152**, 339.

utilized as an approach to the solution of the problem? A number of authors answer the question in the affirmative.[20-25]

Since many of the findings of the above mentioned authors could not be confirmed in this laboratory it was decided to reexamine the problem of the histochemical specificity of phosphatases on a broader basis.

Experimental. The preparation of tissue sections of mouse, rat, guinea pig, dog and human material was essentially the same as reported previously.[26] Six to 20 different tissues of material fixed in chilled alcohol or acetone were embedded in a single paraffin block. In view of the large molecular size of some of the substrates the slides were not coated with collodion. They were incubated for 1 to 24 hr.; phosphate precipitates were visualized by the sulfide technique. The following 19 substrates were used: Metaphosphate; pyrophosphate; methyl-, glycero- and aminoethylphosphate; phenyl, o-chlorophenyl, α-naphthyl-, resorcyl and phenolphthalein-phosphate; glucose-1-phosphate, hexosediphosphate, adenosinetriphosphate, yeast nucleate and thymonucleate; phosphorylcholine and lecithine; octanoylphosphate; p-chloranilidophosphonate. Phosphoric esters not available on the market were synthesized by esterification with $POCl_3$;[27] p-chloranilidophosphonate was prepared by the method of Otto;[28] octanoylphosphate was the gift of Dr. A. Lehninger of the Department of Biochemistry. The concentrations of the substrates ranged from 0.02 to 0.005M or, in the case of nucleates and lecithin, from 0.1 to 0.5%. Experiments were performed at pH5 (acetate buffer), pH7 (tris(hydroxymethyl)-aminomethane-maleate buffer)[29] and pH9 (2-amino-2-methyl-1,3-propanediol buffer).[30] The cation used to trap the phosphate ions was Ca at pH9 and Pb at pH5 and 7. In most cases the concentration of Ca was 0.01M; that of Pb, 0.003M. In some cases, owing to special conditions of solubility, the concentration of the cation had to be changed. For instance, the highly insoluble meta- and pyrophosphates of Ca and Pb will go into solution in the presence of an excess of PO_3^- or $P_2O_7^{\equiv}$ ions. Therefore, a dilute solution of $CaCl_2$ or of Pb $(NO_3)_2$, respectively, was added drop by drop, with constant stirring, to the buffered substrate, until a slight permanent turbidity resulted. This was centrifuged off, and the clear supernatant was used. In other cases, as with phenyl-, o-chlorophenyl- and naphthylphosphate, phosphorylcholine, adenosinetriphosphate, nucleic acids and lecithin, the solubility of the Pb salts (in the case of lecithin and nucleic acids, even that of the Ca salts) proved to be so low that only a very small amount of cation was tolerated without precipitation. It is felt that some of the unsatisfactory results obtained with this latter group of substrates at pH5 and 7 were due to the very poor solubility of the Pb salts of the esters.

Some substrates (octanoylphosphate, adenosinetriphosphate, chloranilidophosphonate) have a slow rate of spontaneous hydrolysis under the conditions of the histochemical experiment. To avoid indiscriminate precipitation of phosphate in the tissues, the Coplin jars were supported in an inclined position, and the slides were placed in them with the sections facing downward. In this way the precipitate collected on the back surface of the slides.

Results. Pyrophosphate did not give positive results at any pH. With metaphosphate a minimal reaction was obtained on the sur-

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30 Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 33.

face of intestinal villi at pH9, otherwise the results were negative. With both substrates spurious reactions consisting of random staining of various tissue elements were seen in all pH ranges. The non-enzymatic nature of this staining could be proven by its presence in inactivated sections, treated for 5 min. with Lugol's solution prior to incubation. Otherwise at pH9 the pictures obtained with all substrates were identical as far as the distribution of the enzyme in the tissues and the relative intensity of the staining at various sites is concerned. Occasionally slides incubated with nucleates for over 5h showed a somewhat more pronounced staining of nuclei than that seen with glycerophosphate as a substrate. However, this difference was neither constant nor conspicuous. Methylphosphate, p-chloranilidophosphonate and especially lecithin were hydrolyzed very much slower than the rest of the substrates; pictures obtained in 24h with the three substrates mentioned were comparable in intensity to those obtained in 1h with the rest. Since no reaction different from that seen with glycerophosphate was observed in the liver with hexosediphosphate or in the muscle with adenosinetriphosphate, it must be assumed that hexosediPh and adenosinetriPh do not survive the embedding procedure.

At pH5 the results were variable. Satisfactory and constant pictures were obtained only with glycerophosphate and resorcinolphosphate, and only after acetone fixation. Alcohol fixation often resulted in patchy, uneven staining. With all other substrates the intensity of the staining was very much lower and, in addition, the results were capricious, even consecutive serial sections showing differences in staining. In the case of nucleate or lecithin as substrates there was often a nonspecific impregnation of mucin, random groups of nuclei, nerve and muscle fibers. Otherwise, the distribution of the enzyme was identical with all substrates and in all tissues, as far as it could be judged from the often unsatisfactory slides. The only exception was p-chloranilidophosphonate. This substrate produced pictures entirely different from the rest.

At pH7, as could be expected, various combination pictures of the typical distribution of acid and alkaline Phs were obtained. The picture normally seen at pH5 showed up as a fairly strong component only when glycerophosphate or resorcinolphosphate were used as substrates, and only after acetone fixation. With the other substrates and/or alcohol fixation the distribution of the enzyme in all tissues was identical with that of alkaline Ph, although the intensity of the staining was much lower. Artifacts consisting of non-enzymatic impregnation of various structures were even more marked than at pH5. Otherwise the results were the same with all substrates. Specifically, the differences due to variation of the substrate, reported by Dempsey and Wislocki,[23] could not be confirmed in either human or guinea pig material.

Since there are reports on the specific inhibitory effects of taurocholate[15] and of cyanide,[24] a number of slides were incubated at pH9 in the presence of these substances, glycerophosphate being used as a substrate. Taurocholate in concentrations from 0.002 to 0.01M produced no visible effect in any of the tissues. Cyanide in concentration from 0.001 to 0.01M caused a marked inhibition of enzymatic activity in all organs. In some cases pictures comparable to those published by Emmel[24] were obtained; in other cases an intense staining persisted in the intestine in the presence of 0.01M NaCN while at the same time the reaction in the kidney was wiped out completely. All possible transitions between these two extremes could be observed in slides carrying a number of kidneys and pieces of small intestine from several animals.

Comment. The dependence of enzymatic behavior on the presence of various colloid substances is well known.[31,32] Even purified enzymes may show widely varying pH optima with different substrates.[33] Owing to these and other factors, the decision on the identity or nonidentity of enzymes may be an exceed-

31 Rona, P., and Gyotoku, K., *Biochem. Z.*, 1926, **167**, 171.

32 Mendel, B., and Rudney, H., *Science*, 1944, **100**, 499.

33 King, E. J., and Delory, G. E., *Biochem. J.*, 1939, **33**, 1185.

TABLE I.

Comparison of Results Obtained with Various Substrates.

Six to 20 different tissues of humans, dogs, rats, mice, and guinea pigs mounted on each slide. Pattern of distribution of enzymatic activity in any given organ is constant and independent of the substrate used. All-over intensity of reaction denoted by plus signs. In the column of pH7, *5* indicates the normal distribution as seen at pH5, *9* that seen at pH9, glycerophosphate being used as a substrate. ? stands for unsatisfactory results.

Substrate	pH5	pH9	pH7
Glycerophosphate	+++	+++	*9* + to ++; *5* + to ++
Pyrophosphate	—	—	—
Metaphosphate	—	—	—
Methylphosphate	—	+	—
Aminoethylphosphate	—	++ to +++	— to *9* +
Phenylphosphate			
o-Chlorophenylphosphate	— to +	++ to +++	*9* + ?
α-Naphthylphosphate	?		
Phenolphthaleinphosphate			
Resorcylphosphate	+++	+++	*9* + to ++; *5* + to ++
Glucose-1-phosphate	—	+++	—
Hexosediphosphate	— to + ?	+++	— to *9* +
Adenosinetriphosphate	—	+++	—
Yeast nucleate	+ to ++	++ to +++	*9* + to ++; *5* +
Thymonucleate	— to + ?	++	*9* +
Phosphorylcholine	—	++	not done
Lecithine	—	+	,, ,,
Octanoylphosphate	not done	++	,, ,,
p-Chloranilidophosphonate	special	+	*9* +

ingly difficult one, even if quantitative chemical procedures are used. The histochemical approach to the problem is beset with the danger of additional sources of error among which only a few will be mentioned.

1. The two main factors regulating the deposition of poorly soluble phosphates in tissue sections are the rates of phosphate production and of diffusion. Varying ratios between these two factors may result in an "all or none" effect, depending on whether the rate of phosphate production will or will not overtake that of diffusion to a point where the solubility product of the phosphate in question is exceeded locally. Any kind of inhibition of enzymatic action (partial inactivation of the enzyme by unsuitable fixation; non-optimal pH or substrate; the presence of various inhibitors) is likely to obliterate areas below a critical level of activity, while areas of much higher activity may remain apparently uninfluenced or only slightly weakened.

2. In the case of substrates of large molecular size the different rates of diffusion into various tissue elements may be a decisive factor.

3. The nonenzymatic impregnation of tissue structures by Pb is well known.[34] The danger of such impregnation is especially great when, on account of long periods of incubation, spontaneous hydrolysis of the substrate may lead to supersaturation in respect to Pb (possibly also of Ca) phosphate. Such artifacts may be recognized by simultaneously incubating inactivated tissues as controls.

Since some of the complications of the histochemical technic cannot be avoided, great caution is warranted in the evaluation of its results, especially when non-optimal conditions are used. Only marked and uniform differences in the picture of enzymatic distribution, obtained by changing conditions such as substrate, pH or inhibitors and activators, can be accepted as a presumptive evidence for the presence of different enzymes. Results of this type have been reported in the case of acid Ph,[35] of acid adenosinetriPh of grain,[20] and of yeast Phs.[25] The importance of such minor differences as reported by Dempsey and Singer,[21] Dempsey and Deane,[22] Dempsey and Wislocki[23] and by Emmel[24] is questionable.

Summary. Nineteen different substrates

[34] Lassek, A. M., *Stain Techn.*, 1947, **22**, 133.

[35] Gomori, G., *Arch. Path.*, 1941, **32**, 189.

were used in this study on the histochemical specificity of phosphatases. With 18 substrates no indication of the presence in paraffin-embedded mammalian tissues of phosphatases other than the common non-specific alkaline and acid variety was found. With one of the substrates, p-chloranilidophosphonate, a strikingly different picture of enzymatic distribution was observed in the acid range, but otherwise the pattern of distribution of enzymatic activity in any given organ was constant and independent of the substrate used.

16808

Effect of Tetraethylammonium on Venous and Arterial Pressure in Congestive Heart Failure.

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That widespread vasoconstriction occurs during congestive failure was first proposed many years ago by Starling[1] and by Bolton.[2] Recently, it has been suggested that reflex vasoconstriction contributes to the maintenance of the usually normal arterial pressure[3,4] and the elevated venous pressure[3,5,6] in heart failure. Conversely, the fall in calculated peripheral resistance accompanying the improved output of the digitalized failing heart[7,8] speaks for a release of vasoconstriction. Renin has been found in the renal veins of patients in congestive failure,[3] and the reduction of hepatic and renal blood flows in such cases is further evidence of increased peripheral resistance attributable to vasoconstriction.

Tetraethylammonium chloride is a quaternary ammonium salt which transiently blocks the transmission of impulses through peripheral autonomic ganglia.[9] Hayward[10] has recently observed that tetraethylammonium bromide decreases the venous and arterial pressures in hypertensive patients with compensated or failing circulations. Since peripheral resistance is greatly increased in essential hypertension, it was of interest to observe the effect of this drug on the venous and arterial pressures of normotensive patients with and without failure, in an attempt to delineate the role of vasoconstriction in congestive failure *per se*.

Methods. Venous pressure was measured directly with a No. 18 gauge needle in an antecubital vein, using the sternal angle of the supine patient as a reference point. Arterial blood pressure was measured with a sphygmomanometer and the mean pressure was arbitrarily taken to be the average of the systolic and diastolic pressures. The heart rate was counted at the apex for 30 seconds. After repeated observations of heart rate and pressures had established a constant baseline, tetraethylammonium chloride (TEAC)* in doses of 2 to 6 mg/kg of body

1 Starling, E. H., The Fluids of the Body. W. T. Keener and Co., Chicago, 1909.

2 Bolton, C., *Brit. Med. J.*, 1917, **1**, 642.

3 Merrill, A. J., Morrison, J. L., and Brannon, E. S., *Am. J. Med.*, 1946, **1**, 468.

4 Landis, E. M., Brown, E., Fauteux, M., and Wise, C., *J. Clin. Invest.*, 1946, **25**, 237.

5 Starr, I., and Rawson, A. J., *Am. J. Med. Sci.*, 1940, **199**, 27.

6 Warren, J. V., and Stead, E. A., Jr., *Arch. Int. Med.*, 1944, **73**, 138.

7 Stead, E. A., Jr., Warren, J. V., and Brannon, E. S., *Arch. Int. Med.*, 1948, **81**, 282.

8 Bloomfield, R. A., Rapaport, B., Milnor, J. P., Long, W. K., Mebane, J., and Ellis, L. B., *J. Clin. Invest.*, 1948, **27**, 588.

9 Acheson, G. H., and Moe, G. K., *J. Pharm. and Exp. Therap.*, 1946, **87**, 220.

10 Hayward, G. W., *Lancet*, 1948, **1**, 18.

* Etamon Chloride, Parke-Davis and Company.

weight was injected through a fresh venipuncture over a period of 2 to 4 minutes, and the pertinent observations were continued frequently for 35 to 40 minutes thereafter.

Twenty subjects were studied in the manner described. Group I contained 8 patients with normal arterial and venous pressures, without evidence of cardiovascular disease. Group II included 2 compensated normotensive cardiacs with normal venous pressures and circulation times. Group III consisted of 8 patients with congestive heart failure as evidenced by distended neck veins, basilar rales, enlarged liver, and (except for patient G.H.) edema. None of these patients had a diastolic blood pressure above 90, and all of them, except G.H., were taking digitalis at the time of the study. Group IV consisted of 2 other patients with congestive failure in whom responses were compared before and after effective treatment for congestive failure. One of these patients (W.G.) had slight diastolic hypertension.

Results. The results are summarized in Table I.

Groups I and II. Venous pressures in the normal subjects and compensated cardiacs did not change, or rose slightly. Results were similar when the pressure in the antecubital vein was artificially elevated to levels seen in congestive failure by lowering the arm. Mean arterial blood pressure fell an average of 2.8 mm Hg $\pm$ 10.9 mm and the pulse rate always increased. The results were the same in normal subjects placed in a semi-reclining position 60 degrees from the horizontal.

Group III. Following injection of TEAC, venous pressure of patients in congestive failure promptly dropped 35 to 80 mm below the baseline. The effect began one to 2 minutes after injection was started, when 100 to 200 mg of the drug had entered the circulation, and was maximal in 5 to 7 minutes. The pressure then started to rise slowly, but usually had not reached its pre-injection level at the end of half an hour. Although there was some overlapping, the average drop in mean arterial blood pressure (24.6 mm Hg $\pm$ 5.6 mm) in Group III was significantly greater than the change seen in Groups I and II ("t" equals 4.25; "P" equals < 0.01). In contrast to the normals, 5 of the 8 patients in Group III had no change in heart rate, 2 slowed slightly, and only one (E.B.) developed a tachycardia. In this patient, a young man with calcified constrictive pericarditis, a tachycardia of the same degree was produced by 1 mg of atropine intravenously, without any change in venous pressure.

Group IV. Two patients in congestive failure were studied before and after digitalization. Before treatment, the injection of TEAC was followed by a sharp fall in venous pressure and a drop in mean arterial pressure. Several days later, when both patients had recovered considerably, the experiment was repeated. In W.G., who had become free of congestive signs and symptoms, TEAC caused no fall in venous pressure. In patient A. H., who still had dyspnea on exertion and basal pulmonary rales, TEAC caused a slight fall in venous pressure. In both patients, the percentile drop in mean arterial pressure was equivalent to that before digitalization.

Arm-to-tongue circulation times, measured with Decholin in 5 subjects before and immediately after injection of TEAC, did not change consistently. No untoward reactions to TEAC were observed, and no change in the degree of the cardiac patients' dyspnea was apparent.

Discussion. In high concentrations, tetraethylammonium increases the cardiac output in a failing heart-lung preparation.[11] However, the drug does not increase the output in dogs under barbiturate anesthesia, and it increases the human cardiac output only slightly or not at all.[12] Moe believes that any increases in output are probably attributable to the tachycardia.

Because the action of TEAC is chiefly on the peripheral autonomic ganglia, the data here presented imply the existence of an increased autonomic vascular tone in congestive failure which contributes to the elevation of venous pressure as well as the maintenance of arterial pressure. Almost the entire peripheral

[11] Acheson, G. H., and Moe, G. K., *J. Pharm. and Exp. Therap.*, 1945, **84**, 189.

[12] Moe, G. K., personal communication.

TABLE I.

Pt., age and sex	Diagnosis*	Dose TEAC, mg/kg	Venous pressure, mm 5% glucose: Baseline corrected to sternal angle	Venous pressure, mm 5% glucose: Max. change	Arterial pressure, mm Hg: Baseline B.P.	Arterial pressure, mm Hg: Mean B.P.	Arterial pressure, mm Hg: Max. change in mean B.P.	Heart rate, per min.: Baseline	Heart rate, per min.: Max. change
Group I									
F.E. 24 M	Normal	6	300†	0	120/72	96	+ 3	74	+ 6
G.G. 25 M	”	6	0	0	128/83	106	0	70	+36
D.F. 24 M	”	6	0	+ 10	110/75	93	— 8	80	+32
M.W. 18 F	Latent syphilis	4	80	0	130/82	106	—16	100	+12
D.B. 48 M	Peptic ulcer	6	78	+ 10	142/84	113	— 5	80	+16
V.C. 29 F	Hemorrhoids	6	135	+ 10	116/76	96	— 4	112	+32
A.Mc. 31 M	Early syphilis	6	30	+ 10	118/72	95	+16	62	+52
O.B.‡ 21 M	” ”	6	10	+ 5	158/66	112	0	80	+60
Group II									
M.C. 18 M	RHD, MS, AS, AI	6	35	0	120/36	78	+ 7	76	+24
G.F. 75 M	ASHD, AF	6	95	+ 15	148/64	106	—21	74	+14
		Mean:	51.5 ± 38.8	+6.6 ± 5.8		100.1 ± 10.4	—2.8 ± 10.9		
Group III									
G.H. 54 M	ASHD, My	2	110	— 55	96/60	78	—10	98	— 8
E.B. 24 M	CP, AF	3	165	— 65	110/80	95	—21	84	+22
J.Y. 58 M	CP, AF	6	200	— 70	126/68	97	—27	74	0
W.H. 64 M	SA, AI	4	25	— 35	180/87	134	—19	80	0
M.M. 61 M	RHD, MS, MI, AI, TI, AF	6	80	— 80	128/65	97	—25	62	0
J.W. 49 M	ASHD, AF, ?RHD, ?TI	6	195	— 75	100/76	88	—18	90	0
A.E. 47 M	RHD, MS, AS, AI, AF	6	80	— 45	170/60	115	—31	68	—10
J.D. 65 M	ASHD, ?CP	6	65	— 70	110/78	94	—46	92	0
		Mean:	115 ± 64.5	—61.9 ± 15.6		99.8 ± 17.2	—24.6 ± 10.7		
Group IV									
W.G. 31 M	HHD, ASHD (before digitalis)	3.6	240	—120	146/120	133	—29	130	+36
W.G.	(After digitalis)	4.2	60	+ 25	130/96	113	—21	98	0
A.H. 66 M	ASHD (before digitalis)	4.5	155	— 90	134/82	108	—60	112	0
A.H.	(After digitalis)	5.4	—25	— 20	118/80	99	—53	108	0

* RHD—Rheumatic Heart Disease. ASHD—Arteriosclerotic Heart Disease. HHD—Hypertensive Heart Disease. SA—Syphilitic Aortitis. MS—Mitral Stenosis. AS—Aortic Stenosis. AI—Aortic Insufficiency. TI—Tricuspid Insufficiency. CP—Constrictive Pericarditis. My—Myocardial Infarction.

† The arm was lowered far below the level of the heart, but no correction to the level of the sternal angle was made. This value is not included in the calculation of the mean.

‡ This patient had received 0.5 mg of adrenalin subcutaneously 40 minutes before these observations.

vascular bed is under active vasomotor control.[13] A reduction in the amount of blood pumped from the venous to the arterial side of the circulation would lower arterial pressure while tending to elevate venous pressure.[1,2,5] It seems possible, therefore, that whenever cardiac output is inadequate, reflex peripheral autonomic mechanisms are called into play. These would maintain arterial pressure by arteriolar constriction and tend to increase cardiac output by further increasing the venous filling pressure. Venous filling pressure might be elevated by an increased tone in the large veins or by a redistribution of blood resulting from constriction of smaller peripheral vessels. These experiments do not exclude the possibility that other factors such as increased blood volume or tissue fluid pressure may also play major roles in the maintenance of the elevated venous pressure of congestive failure.

Finally, it should be pointed out that only the peripheral venous pressure was measured in these experiments. It is possible, particularly in subjects without venous hypertension, that pressure changes in the central veins and right atrium were not closely reflected by changes in the peripheral venous pressure.

Summary. Tetraethylammonium chloride given intravenously to 9 normotensive patients in congestive failure caused a precipitous fall in venous pressure and a significant decrease in arterial pressure in every case. Compensated cardiacs and non-cardiac normotensive controls showed no fall in venous pressure and a much smaller change in arterial pressure. Following digitalization, 2 cardiac subjects in failure lost some of their initial responsiveness to TEAC.

[13] McDowall, R. J. S., *Phys. Rev.*, 1935, **15**, 98.

16809

Survival of Rats After Temporary Complete Renal Ischemia.*

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It has been shown that rats generally survive bilateral interruption of the renal circulation for periods lasting up to one hour.[1] With longer intervals of complete ischemia, the mortality rises sharply. Cessation of blood flow to both kidneys for two hours is uniformly fatal; there is progressive elevation of BUN and death occurs in uremia within a few days.

The principal lesion produced by temporary periods of complete renal ischemia is necrosis of the proximal convoluted tubules.[1] In rats with a one hour period of bilateral ischemia, the necrosis is followed by repair and survival of the animals. Rats with a 2 hour period of ischemia evidently die in uremia before regeneration is effective.

In order to determine whether the renal lesion produced by periods of ischemia longer than those in the preceding experiments was subject to repair and would permit survival of the rats, unilateral ischemia was produced. The left main renal artery and vein of 50 adult white rats were occluded with a bulldog clamp for periods ranging from 2 to 4 hours. A 5 hour period was unsatisfactory because of the frequent occurrence of renal infarction. Heparin was given intravenously before clamping, and in some instances during clamping, to retard intrarenal thrombosis. The right kidney was intact. The animals were sacrificed at various intervals up to 5 months after removal of the clamps.

Results. Tubular necrosis was followed by

* Aided by a grant from the Elisabeth Severance Prentiss Foundation.

[1] Koletsky, Simon and Gustafson, G. E., *J. Clin. Invest.*, 1947, **26**, 1072.

TABLE I.
Mortality in 75 Rats After Complete Ischemia of Left Kidney and Subsequent Resection of the Right Kidney.

Duration of left renal ischemia, hrs	No. of rats	No. of rats which died	Mortality, %
1½	15	0	0
2	15	1	7
3	15	6	40
3½	15	9	60
4	15	13	87

repair in all rats. At the end of one week the tubules were largely cleared of necrotic débris and were lined by a newly formed epithelium. However after one week there was progressive and profound renal atrophy so that at 3 weeks the kidney, although completely repaired, was reduced to approximately one half to one third the normal size. Both glomeruli and tubules were atrophic and the interstitial fibrous stroma increased in amount. The tubular lumens were narrow or closed and the lining cells small. Further observations showed that the atrophy resulting from temporary ischemia persisted indefinitely as long as the complete opposite kidney was intact.

The functional capacity of the small atrophic kidney resulting from temporary interruption of blood flow was then investigated. Clamps were applied to the left main renal artery and vein of 75 adult rats divided into 5 groups of 15 rats each, with the following periods of occlusion respectively, *i.e.*, 90 minutes and 2, 3, 3½, and 4 hours. The right kidney was resected 3 weeks after removal of the clamp. BUN was determined by the method of Ormsby[2] before and after release of the clamps and at regular intervals following resection of the right kidney. Heart's blood was obtained for BUN in rats which died in uremia. Surviving animals were sacrificed from 3 to 5 months after the right kidney was removed.

Results. The mortality among the 5 groups of rats is shown in Table I. In the rats which died the BUN usually rose rapidly and death occurred within 1 or 2 weeks after resection of the right kidney. Terminal BUN values ranged from 220 to 764. Four animals developed chronic uremia with BUN levels up to 300 and marked weight loss, and these died from 4 to 11 weeks after resection of the right kidney.

All rats in the 90 minute group and 14 of 15 in the 2 hour group survived. In the 3 and 3½ hour groups, 9 and 6 animals survived respectively as compared to only 2 survivals in the 4 hour group. All surviving rats were in good condition and either maintained or gained weight. However, except for most animals in the 90 minute and 2 hour group, the BUN subsequent to resection of the right kidney was permanently elevated. The levels were usually below 100 mg per 100 cc with ischemia up to 3 hours' duration and above 100 in the animals with 3½ and 4 hour periods of ischemia. Two of the 6 surviving rats with 3½ hour renal ischemia, after being in good condition for 4 months, had a sudden rise in BUN and developed uremia. At autopsy the kidneys were the seat of necrotizing arteriolitis, especially the glomeruli, and also acute pyelonephritis.

In all surviving rats resection of the right kidney was followed by marked compensatory hypertrophy of the left kidney. The latter was large, usually exceeding normal size, and both glomeruli and tubules were hypertrophic. In contrast the rats which died following removal of the right kidney had a small atrophic left kidney with minimal or no compensatory hypertrophy.

Conclusion. These experiments indicate that if the period of complete ischemia does not exceed 2 hours, the rat kidney can regain enough function to permit survival. With complete ischemia of 3 hours' duration chance of survival is reduced to about half. Cessation of renal blood flow for more than 3 hours usually results in loss of capacity for compensatory hypertrophy and hence may be considered irreversible.

2 Ormsby, A. A., *J. Biol. Chem.*, 1942, **146**, 595.

16810

Electroencephalograms in Behavior Changes in Cats.

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Lesions of the ventromedial hypothalamic nuclei in cats usually are accompanied by striking behavior changes, depending upon the symmetry and completeness of these rather small lesions (Wheatley[1]). Postoperatively these animals display a malevolent attitude, are extremely difficult to handle and thereafter are quite untameable. Hyperphagia is frequently part of this syndrome and the animals may become very obese. There are no motor defects and their behavior is well-controlled and purposeful, not to be compared with the sham rage of decorticate preparations. How these small, subcortical lesions produce such effects has not been explained, although a disturbance in hypothalamo-cortical relationships is presumably involved. Since clinical observations[2–11] have indicated that a high proportion of patients showing behavior disorders may have abnormal electroencephalograms, and since Hoagland[2] observed that emotional stimulation may increase delta activity from both hypothalamus and cortex, it was thought that electroencephalographic observations on these cats might indicate some physiological change in cortical activity.

The bilateral lesions were produced by electrolysis, and in all cases of savage behavior were found to be restricted to the immediate vicinity of the ventromedial nuclei. All cats used were in good health at the time of experiment. The experiments were performed as follows: Fifteen mg of beta erythroidin hydrobromide in 0.9% NaCl solution were injected intravenously, and an intratracheal catheter inserted. Artificial respiration was maintained by positive pressure, the respirator being set to provide 27 inspirations a minute. Complete paralysis was maintained by the administration of a solution of erythroidin containing 0.5 mg per cc, given intravenously at a suitable rate. Our experience, in agreement with that of others,[12] indicates that such curarization does not significantly affect the EEG. Silver electrodes were fixed to the scalp with electrode paste and collodion. The numerical designation and position of the leads were: 1, occipital; 2, parietal (vertex); 3, frontal; 4, the common lead, was from the ear. Some of the resistance offered by skull, skin, etc., was eliminated by removal of bone from the skull areas beneath the electrode sites a week or more before the first run. Each animal was subjected to several twenty minute periods of recording. A six channel Offner electroencephalograph with a crystograph recorder was used.

Observations. Records from over a hundred experiments of this type are in general agreement with those of Clark and Ward[13] on normal cats with implanted leads and with similar preparations of our own. Normal

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2 Hoagland, H., *J. Gen. Psychol.*, 1938, **19**, 227.

3 Jasper, H. H., Solomon, P., and Bradley, C., *Am. J. Psych.*, 1938, **95**, 641.

4 Lindsley, D. B., Cutts, K. K., *Arch. Neurol. and Psychiat.*, 1940, **44**, 1199.

5 Knott, J. R., and Gottlieb, J. S., *Arch. Neurol. and Psychiat.*, 1944, **52**, 515.

6 Gottlieb, J. S., Knott, J. R., and Ashby, M. C., *Arch. Neurol. and Psychiat.*, 1945, **53**, 138.

7 Michaels, J. J., and Secunda, L., *Am. J. Psychiat.*, 1944, **101**, 407.

8 Michaels, J. J., *Psychosomatic Medicine*, 1945, **7**, 41.

9 Will, O. A., *U. S. Naval Med. Bull.*, 1945, **44**, 341.

10 Simon, D. J., and Diethelm, O., *Arch. Neurol. and Psychiat.*, 1946, **55**, 619.

11 Rockwell, F. V., and Simons, D. J., *Arch. Neurol. and Psychiat.*, 1947, **57**, 71.

12 Girden, E., *J. Neurophysiol.*, 1948, **11**, 169.

13 Clark, S. L., and Ward, J. W., *J. Neurophysiol.*, 1945, **8**, 98.

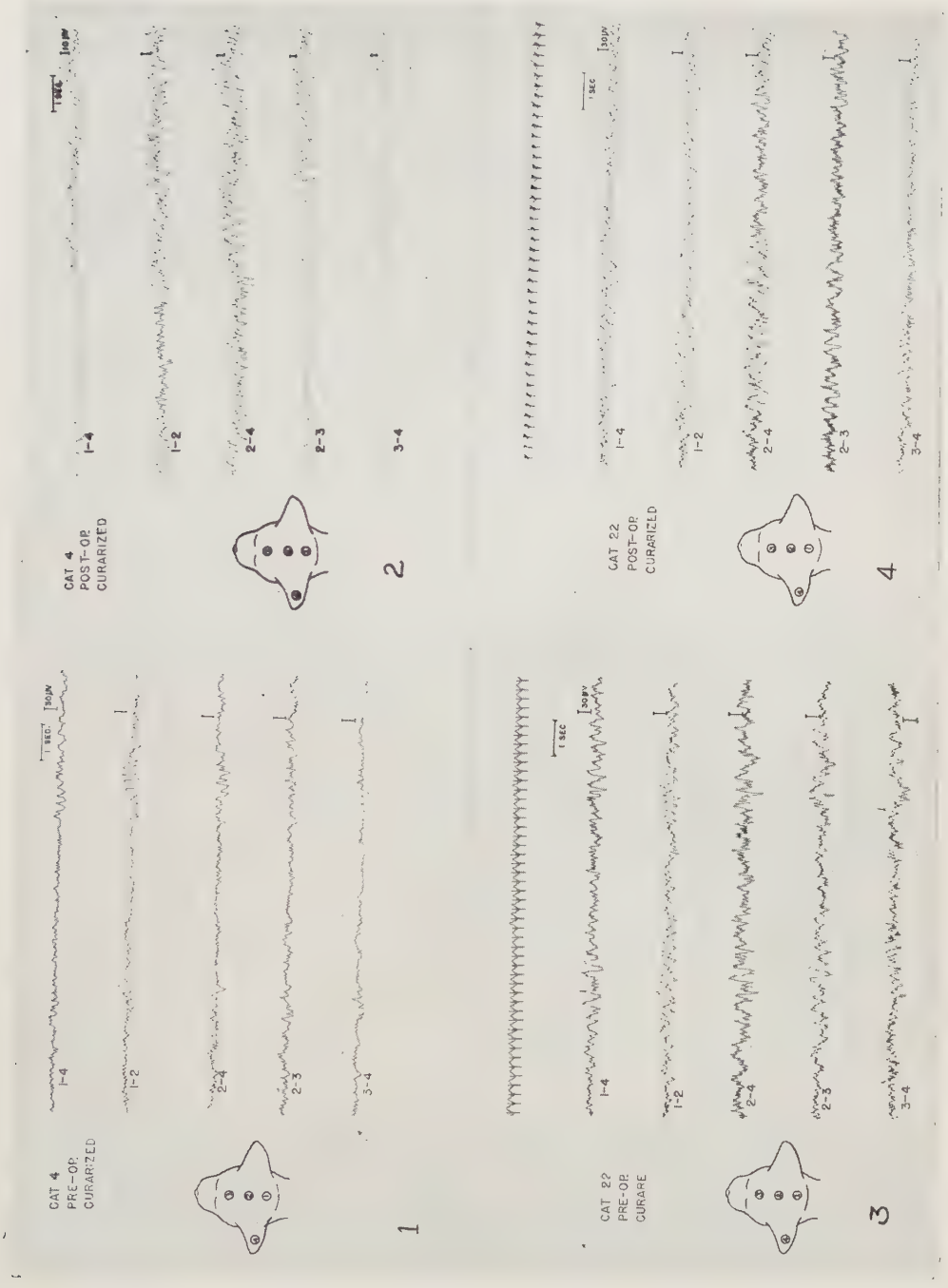

Fig. 1. Preoperative EEG of cat 4, which later became savage.
Fig. 3. Preoperative EEG of cat 22, a control.
Fig. 2. Postoperative EEG of cat 4, in savage state.
Fig. 4. Postoperative EEG of cat 22, a control.

cats show a striking variability in the EEG as to frequency, amplitude and the occurrence of random bursts of fairly uniform frequencies, even when curarized. Differences between normal curarized and non-curarized animals are relatively slight. It is of some interest that EEG patterns resembling those which accompany the appearance of sleep in cats may occur with great frequency under erythroidin. Such patterns may be seen in

Fig. 5.
Graphic representation of frequency analyses for cat 2, savage, and cat 22, control. Each graph represents a 20-minute run. Each vertical bar indicates the percentage of a frequency band in a 30-second sample of the record.

Fig. 1 and 2, especially from the occipital and vertex leads, but less evident from the frontal monopolar lead. Sample records of normal unoperated cats are shown in Fig. 1 and 3. Fig. 2 is a record from the same cat as in Fig. 1, after the animal had become extremely savage following operation for production of hypothalamic lesions. Fig. 4 is a postoperative record from the same cat as in Fig. 3, but in this case no savageness was present.

Inspection of records from normal and savage cats and comparison of preoperative and postoperative records from savage cats disclosed no qualitative differences which were considered to be outside the normal variation. In an attempt at more precise analysis, several 30 second samples from the vertex lead of each run were measured for frequency distribution. Four frequency categories were used: (a) very slow (S_2), less than 3.5/sec.; (b) slow (S_1), 4-8/sec.; (c) intermediate (I), 8-12/sec.; (d) fast (F), more than 12/sec. Examples of the distribution patterns in 2 cats, one savage and one unoperated control are shown in Fig. 5, and a summary of the findings in normal, savage and control cats will be found in Table I. Inspection of these data leads to the conclusion that no significant difference exists. This conclusion is strengthened by the degree of variability in the same animals, pre- and postoperatively, and justifies rejection of the hypothesis that any sig-

TABLE I.
Frequency Analysis of Normal and Operated Cats.
(Lead 2-4.)

Experiments	Avg % frequency			
	S_2	S_1	I	F
29 normal (13 cats)	8.86 ± 1.98*	37.48 ± 2.36	26.18 ± 2.2	27.13 ± 2.79
14 operated controls (5 cats)	3.62 ± 0.84	34.62 ± 4.33	38.5 ± 2.76	23.34 ± 4.64
19 malevolent (5 cats)	9.43 ± 1.64	32.06 ± 2.3	29.27 ± 1.79	30.66 ± 2.74

S_2 indicates frequencies less than 3.5 per second; S_1 indicates frequencies of 4-8 per second; I indicates frequencies of 8-12 per second; F indicates frequencies greater than 12 per second.
* Standard error.

nificant trends exist after this type of hypothalamic injury.

Corticohypothalamic relationships remain physiologically and anatomically obscure. Technical factors have interfered with anatomical studies (Ingram[14]). Neuronographic studies (Ward and McCulloch[15]; Murphy and Gellhorn[16]) indicate that connections between the frontal lobe, at least, and hypothalamus are fairly extensive. The significance of these connections is not clear. According to Kennard[17] and Obrador,[18] extensive destruction of the hypothalamus suppresses cortical potentials. On the other hand, Morrison, *et al.*[19] found no relationship between hypothalamic and cortical activity, and Jasper and Droogleever-Fortuyn[20] found no generalized modification of cortical potentials upon stimulation of the hypothalamus. Laufer[21] and Lennox and Brody[22] have reported that hypothalamic lesions in human patients are associated with generalized low frequency activity. Our own results indicate that relatively small, bilateral lesions restricted to the ventromedial part of the tuber do not alter cortical activity in curarized animals, even when such lesions may be considered causally related to decided changes in behavior with outward signs of an emotional type. However, records from the cortices of such animals under more nearly "normal" circumstances, unaffected by drugs, both at rest and when displaying their characteristic rage, have not yet been secured, and such data will be an essential part of the final interpretative picture. Under aggravation, savage cats show violent autonomic discharges, which are not detectable under erythroidin. Under non-narcotized conditions Darrow's[23] theory that such discharges may be associated with changes in cortical activity may then well be put to test.

Summary. Electroencephalographic studies of cats curarized with erythroidin show no discernible differences between cortical potential patterns in normals, operated controls and cats showing "savage" behavior after production of relatively specific hypothalamic lesions.

14 Ingram, W. R., *Res. Publ. Ass. nerv. ment. Dis.*, 1940, **20**, 195.

15 Ward, A. A., and McCulloch, W. S., *J. Neurophysiol.*, 1947, **10**, 310.

16 Murphy, J. P., and Gellhorn, E., *J. Neurophysiol.*, 1945, **8**, 431.

17 Kennard, M. A., *J. Neurophysiol.*, 1943, **6**, 233.

18 Obrador Alcalde, S., *J. Neurophysiol.*, 1943, **6**, 81.

19 Morison, R. S., Finley, K. H., and Lothrop, G. N., *J. Neurophysiol.*, 1943, **6**, 243.

20 Jasper, H. H., and Droogleever-Fortuyn, J., *Res. Publ. Ass. nerv. ment. Dis.*, 1947, **26**, 272.

21 Laufer, M. W., *J. Nerv. and Ment. Dis.*, 1947, **106**, 527.

22 Lennox, M., and Brody, B. S., *J. Nerv. and Ment. Dis.*, 1946, **104**, 237.

23 Darrow, C. W., *Am. J. Psychiat.*, 1945, **102**, 791.

16811

Studies on the Blood Pyridoxine of Vitamin B_6 Deficient Monkeys.*

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Past experience has shown that in general the vitamin content of the blood is influenced by the quantity in the diet. In the case of vitamin C, thiamin and pantothenic acid, it is possible to use the blood level as a criterion of the adequacy of the intake. When this work was commenced, no reports on the concentration of the pyridoxine in blood of any species were to be found in the literature. Since then Ritchey *et al.*[1] using an assay with Neurospora have reported a value of 42 μg per 100 ml for pooled mouse blood. Subsequently Ray and co-workers[2] using a modification of the yeast method of Atkins and associates,[3] presented data on the blood pyridoxine content of control and cobalt deficient sheep. Employing a modification of the yeast method of Atkins and associates, our laboratory has carried out a rather extensive study of the blood pyridoxine in monkeys and humans and has studied its distribution in plasma and erythrocytes.

The present paper deals with the blood pyridoxine levels in monkeys and its alteration during deficiency of this vitamin. To date observations have been made on 14 deficient monkeys. A few of our early results were presented along with a description of the early manifestations of pyridoxine deficiency at the Federation meetings in 1946.[4] Since then our study has been greatly extended.

Experimental. Young rhesus monkeys weighing between 1.8 and 3.0 kg were used in these experiments. The methods employed in handling and feeding the animals have been described in an earlier publication.[5] The basal diet was a modified M-3 diet[6] and contained powdered sucrose 73, vitamin-free casein (GBI or Labco) 18, Hawk and Oser salt mixture 4, and corn oil 2. It was compressed into 2 g tablets on a Stokes tablet machine following granulation and the addition of 1% calcium stearate as a lubricant. The tablets were fed *ad libitum*. A vitamin tablet containing the following was fed daily; Nicotinic acid 5 mg, riboflavin 1 mg, thiamin chloride 0.5 mg, calcium pantothenate 3 mg, choline dihydrogen citrate 100 mg, para-aminobenzoic acid 100 mg, inositol 100 mg, ascorbic acid 25 mg, plus sufficient powdered sugar to make a tablet weighing 1.5 g. In addition the monkeys received by mouth twice weekly 5 drops vitamin A and D concentrate (100,000 I. U. vitamin A and 10,000 I. U. vitamin D per g), 385 μg† pteroylglutamic

* This investigation was aided by grants from the California Fruit Growers Exchange and the Christine Breon Fund for Medical Research. We are grateful to Merck and Co., Rahway, N. J., for supplies of biotin and inositol; to Lederle Laboratories, Pearl River, N. Y., for pteroylglutamic acid; and to Mr. Stephen Dean of the College of Pharmacy for assistance in the preparation of diet tablets.

1 Ritchey, M. G., Wicks, L. F., and Tatum, E. L., *J. Biol. Chem.*, 1947, **171**, 51.

2 Ray, S. N., Weir, W. C., Pope, A. L., and Phillips, P. H., *J. Nutrition*, 1947, **34**, 595.

3 Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Indust. and Engr. Chem. Anal. Ed.*, 1943, **15**, 141.

4 Greenberg, L. D., and Rinehart, J. F., *Fed. Proc.*, 1946, **5**, 222.

5 Rinehart, J. F., Greenberg, L. D., and Ginzton, L. L., *Blood*, 1948, in press.

6 Waisman, H. A., and McCall, K. B., *Arch. Biochem.*, 1944, **4**, 265.

† Two of the monkeys were started on one-half this intake of pteroylglutamic acid. This is equivalent to a daily dose of 55 μg and was originally reported to be adequate by Totter and associates.[10] Dr. Totter had later informed us that this intake was not adequate, so we increased it to 110 μg per day and we have found this intake to be adequate.

10 Totter, J. R., Shukers, C. F., Kolson, J., Minus, V., and Day, P. L., *J. Biol. Chem.*, 1944, **152**, 147.

acid, 10 micrograms biotin and 5 drops of mixed natural tocopherols (Nopco) once a week. Control monkeys were also provided with 3.5 mg of pyridoxine hydrochloride twice a week.

During the course of these studies careful records of the semi-weekly weights and of the daily food consumption were kept. Generally, blood was taken by venipuncture at weekly or bi-weekly intervals and used for total blood counts, for serum iron and pyridoxine analyses. At autopsy, portions of several tissues were removed for vitamin assay. The monkeys were usually placed on the diet with complete supplements for 2 to 4 weeks before withdrawal of the pyridoxine, in order to perform control tests so that each animal might serve as his own control. In addition to studies of the pyridoxine levels of the blood, periodic examinations of the metabolism of tryptophane and of carbohydrate were undertaken. Since this paper is to be confined to the studies on the blood pyridoxine levels of the monkeys, it will suffice at present to merely mention the fact that in the pyridoxine deficient monkey, as in the pyridoxine deficient dog,[7] rat,[8] and pig[9] there is a derangement of tryptophane metabolism, resulting in the excretion of xanthurenic acid.

Neurospora assay was found to lack sufficient sensitivity for our work, so we finally settled on the method of Atkins *et al.*[3] with slight modifications. The procedure for the extraction of the vitamin B_6 consisted in adding one volume of blood or plasma to 18 volumes of 0.055N H_2SO_4 (1 ml of 10N H_2SO_4 to 179 cc of H_2O) and autoclaving for 1 hour at 20 lb pressure. After cooling, the pH of the mixture was adjusted to 5.2 and the volume was made up to 25 to 30 times that of the blood used. The precipitated proteins were removed by centrifugation and the supernatant fluid was decanted. Since assay tubes containing the extracts developed turbidity following sterilization as a result of additional precipitation of protein or protein cleavage products, it was found necessary to reheat the extracts either in the autoclave for a period of 5-10 minutes at 15 lb pressure or in flowing steam for 10 minutes and to recentrifuge them. In this manner development of turbidity in the assay tubes could be avoided. The extract was now ready for testing. If the extracts were to be stored they were given a short period of sterilization and kept in the refrigerator until it was convenient to assay them. Because of the destructive effects of light, precautions were taken to keep the exposure to light at a minimum during the preparation and handling of the extracts.

As an alternate method of extraction of the vitamin we have employed takadiastase digestion in a manner similar to that described by Luckey and coworkers[11] for the extraction of pteroylglutamic acid. This has yielded, with few exceptions, substantially the same values as acid extraction. Treatment of the two sets of values by a standard statistical method showed that there was no statistically reliable difference between the data obtained with the two extraction procedures.

The growth of the test micro-organism, *Saccharomyces carlsbergensis* No. 4228, the preparation of the inoculum and the assay procedure were essentially similar to that described in the original method. A minor modification consisted in the incubation of cultures and assay tubes at room temperature (ca 25-26°) instead of at 30°. The extracts were generally assayed at levels of 2, 3 and 4 ml and growth was measured turbidimetrically with the Evelyn colorimeter using filter 620 after 16 and 18 hours of continuous agitation on a Kahn shaker. Since it was found that little was to be gained by the second set of readings, this was discontinued in many of our later analyses. Owing to the fact that extracts of blood contain some color, it was found advisable to take blank readings on the

7 Axebrod, H. E., Morgan, A. F., and Lepkovsky, S., *J. Biol. Chem.*, 1945, **160**, 155.

8 Lepkovsky, S., Roboz, E., and Haagen-Smit, A. J., *J. Biol. Chem.*, 1943, **149**, 195.

9 Cartwright, G. E., Wintrobe, M. M., Jones, P., Lawritsen, M., and Humphreys, S., *Bull. Johns Hopkins Hosp.*, 1944, **75**, 35.

11 Luckey, T. D., Briggs, G. M., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **152**, 157.

assay tubes immediately following inoculation with the yeast.

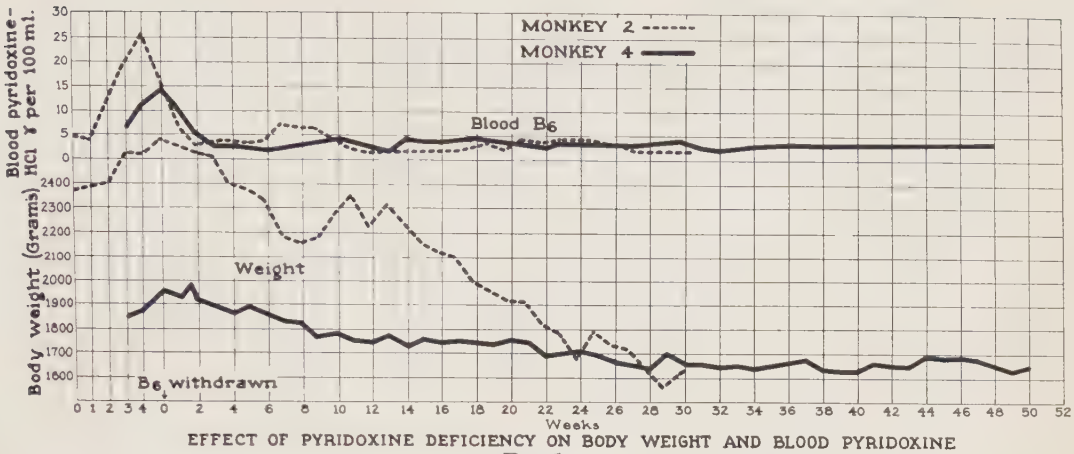
Results. Following control observations on the experimental animals, pyridoxine was withdrawn from the diet and within 2 to 4 weeks after withholding the vitamin the monkeys ate less food and began to lose weight slowly and progressively. In most cases this weight loss continued throughout the experiment. Aside from decreased food consumption, loss in weight and some loss in vigor, the animals showed little change in outward appearance until approximately 6 to 9 months after pyridoxine deprivation when they became unkempt, sluggish in their movements and showed some signs of being hyperirritable. In addition they generally developed edema around the eyes. Similar observations have been made by McCall *et al.*[12] in the monkey. Most of the animals have shown changes in their hair and these have varied considerably from animal to animal. These alterations have consisted of thinning of the hair in some monkeys, of patches of baldness in others, of extensive loss of hair in others, while others have exhibited little change in the appearance of the coat except slight greying. The majority of the monkeys studied have usually developed fissuring and cracking of the epidermis of the hands after some 3 to 6 months of the dietary regime. A constant finding has been the development of a slowly progressive anemia. Details of this will be published later.

During the course of the experiment the pyridoxine content of the blood was followed. It was found that when monkeys were first brought into the laboratory their blood levels were usually below 5 μg per 100 cc of whole blood, but after having been on the diet with complete supplements for 2 to 3 weeks the levels increased to values ranging from 5 to 25 μg per 100 cc. Following withdrawal of the vitamin it was a matter of but a week or two until the pyridoxine of the blood had decreased to values below 5 μg, and 3 to 4 weeks following the time of withdrawal of the vitamin the levels had decreased to the neighborhood of 2 to 3 μg. As the deficiency progressed the values remained in the same range or declined still further except for a few unexplainable fluctuations. Frequently, in long-standing deficiencies we have observed values of the order of 1 μg. Two typical cases which are representative of the alterations occurring in the weight curve and in the pyridoxine content of the blood deficiency are represented graphically in Fig. I. The pyridoxine level of the blood is affected very early and it exhibits a fall before there is any appreciable loss in weight. Fig. 2 and 3 are examples of the dramatic response in the weight, food consumption and pyridoxine levels of the blood of two long-standing pyridoxine deficient monkeys following the administration of the vitamin. Monkey 3 (Fig. 2) received 3.5 mg of pyridoxine hydrochloride twice a week while monkey 1 (Fig. 3) received a supplement of 1 mg daily. The increase in weight following the administration of the vitamin was phenomenal. In addition to the functions represented in the graphs there was a return to normal of all the observed abnormalities such as blood picture, etc. Fig. 2 also shows the fall in the pyridoxine level of the blood following the withholding of the vitamin from the diet for a second time. In this instance also the fall is rapid.

For purposes of comparison the pyridoxine levels of 2 control monkeys are given in Fig. 4. Although there is considerable fluctuation in the blood pyridoxine values, it can be seen that in no case have the values fallen below 5 μg per 100 ml. In a majority of the cases the values have been above 10 μg. In the cases of some rapidly growing monkeys which we have been studying recently we found it necessary to double the pyridoxine intake in order to bring their blood pyridoxine levels above 5 μg.

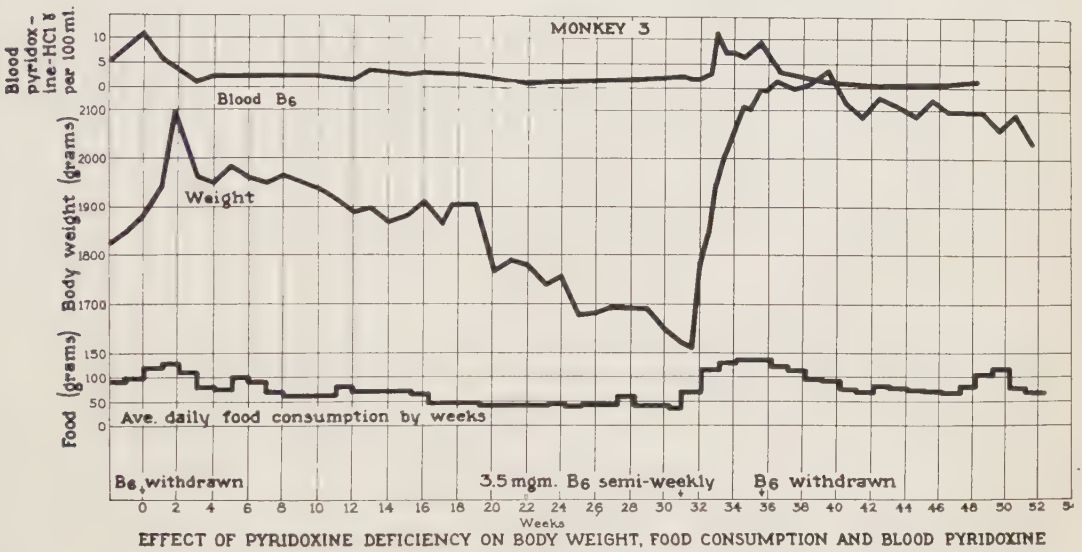
The blood pyridoxine levels of 2 monkeys (A.A. deficient 3360 and 3397) which had been followed before and after deprivation of ascorbic acid are also represented in Fig. 4. The diet and supplements were the same as used for control animals with exception that

[12] McCall, K. B., Waisman, H. A., Elvehjem, C. A., and Jones, E. S., *J. Nutrition*, 1946, **31**, 685.



EFFECT OF PYRIDOXINE DEFICIENCY ON BODY WEIGHT AND BLOOD PYRIDOXINE

FIG. 1.

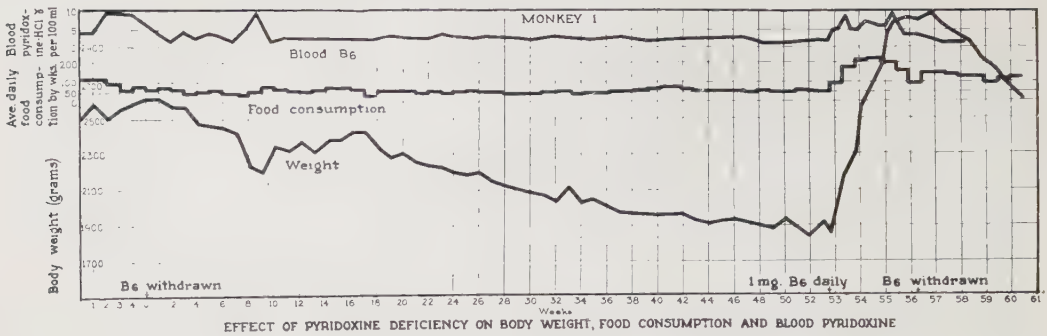


EFFECT OF PYRIDOXINE DEFICIENCY ON BODY WEIGHT, FOOD CONSUMPTION AND BLOOD PYRIDOXINE

FIG. 2.

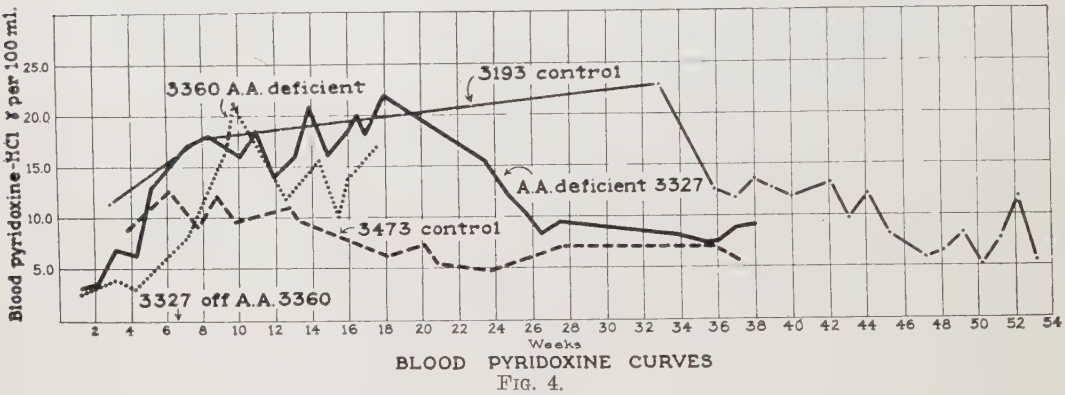
after a period of 44 days ascorbic acid was withdrawn in connection with some other work. The curves are of the same general type as is found in our long-standing control animal (3193). It is significant that when these animals were first brought to the laboratory and placed upon the experimental diet with complete supplements, their blood pyridoxine levels were in the range of approximately 2.5 to 3.0 μg per 100 ml. During the course of 4 to 6 weeks on the diet the pyridoxine values had risen considerably above 5 μg to a range of between 8 and 18 μg. Similar observations have been made with several other monkeys.

In addition to the studies on the pyridoxine of whole blood, the distribution of the vitamin in plasma and red cells has been investigated in several instances. The results which have been obtained to date are summarized in Table I and these show that the vitamin is present in both plasma and erythrocytes. The data in the last 2 columns show that the actual blood pyridoxine concentration is in good agreement with the values calculated from the packed cell-volume, and the plasma



EFFECT OF PYRIDOXINE DEFICIENCY ON BODY WEIGHT, FOOD CONSUMPTION AND BLOOD PYRIDOXINE

FIG. 3.



BLOOD PYRIDOXINE CURVES

FIG. 4.

and erythrocyte concentrations of the vitamin. In monkeys on a good intake the plasma generally shows a higher concentration than the erythrocytes, but in deficient monkeys the values approach nearly equal distribution in plasma and red cells. Alterations in intake appear to influence the plasma concentration more rapidly than the erythrocyte concentration of the vitamin. The distribution of pyridoxine in the blood differs from that of thiamin, since it has been shown that approximately 90 per cent of the latter is located in the red cells. On the other hand there is a great similarity in the distribution of pyridoxine and pantothenic acid in the blood, for Pearson[13] has shown that in all the animals studied, with the exception of man, the plasma had a greater concentration of pantothenic acid than the red cells.

Discussion. The blood pyridoxine values observed are somewhat similar to those found for thiamin in the monkey.[5] The former may reach values somewhat higher than is found with thiamin, when the intakes of the 2 vitamins are of about the same order.

The average value of 42 μg per 100 ml of whole blood obtained upon mice by Ritchey *et al.*[1] is approximately twice as high as the highest values observed in our experiments. On the other hand, the results obtained by Ray and co-workers[2] on sheep are more nearly in the range of values observed by us in the monkey. They obtained an average value of 11.8 μg of pyridoxine per 100 ml of whole blood on control sheep. However, the vitamin B_6 intake of the sheep was not reported. The values on 2 control monkeys have ranged between 5.0 and 20.7 μg and have averaged 11.2 μg per 100 ml for 35 serial determinations. The average of 36 assays on the 2 monkeys used in the ascorbic acid deficiency experiment is 13.6 μg and the range is 6.1 to 21.9 μg per 100 ml of whole blood.

At present we have no adequate explanation for the wide fluctuation in the vitamin

[13] Pearson, P. B., *J. Biol. Chem.*, 1941, **140**, 423.

TABLE I.
Distribution of Pyridoxine in Blood (μg pyridoxine-hydrochloride per 100 ml).

Monkey and specimen No.	Plasma pyridoxine (1)	Red cell pyridoxine (2)	Packed-cell vol. (3)	Whole blood pyridoxine	
				observed	calculated*
3327	8.9	21.1	44	13.7	14.3
6 —a	8.4	4.6	44	5.6	6.7
—b	2.5	3.9	42	3.3	3.1
8 —a	18.2	4.7	41	11.6	12.6
—b	15.3	5.7	41	9.9	11.4
—c	10.1	4.0	42	6.2	7.5
3473	5.5	5.7	47	5.4	5.6
3193 —a	10.6	4.4	42	9.4	8.0
—b	8.3	4.4	40	7.8	6.7
—c	8.0	4.0	37	6.8	6.5
—d	2.6	3.8	37	3.4	3.0
3 D†	1.9	—	—	1.9	—
4 D	2.2	1.8	41	2.3	2.0
1 D	1.2	1.8	42	1.6	1.4
11 D	2.4	3.9	44	2.9	3.1

* Calculated from columns 1, 2, and 3.
† D—Pyridoxine Deficient.

B_6 values of the animals receiving pyridoxine, except to say that it does not appear to be a question of technic, since duplicate assays yield values in close agreement. In view of the fact that Ray *et al.*[2] have shown that the blood pyridoxine levels are lowered in cobalt deficient sheep, it is possible that this fact may have some bearing on the lower values obtained in the 2 monkeys (No. 3327 and No. 3193) after a lapse of some 6 to 8 months. However, this seems rather unlikely since so far only ruminants have been shown to be susceptible to cobalt deficiency. Since the vitamin B_6 intake remained constant, the decrease in the blood pyridoxine concentration would most likely be attributable to the smaller intake of the vitamin on a weight basis resulting from the great increase in size of the animal.

Summary. Suitable modifications for the extraction and assay of blood pyridoxine are described. The vitamin B_6 levels of 14 rhesus monkeys were followed before and after withdrawal of the vitamin from the diet. The vitamin B_6 levels fell during the first 2 weeks of deprivation. This usually brought the levels down to values of 2-3 μg per 100 ml of whole blood or lower, where they remained throughout the rest of the experiment for periods exceeding 1 year. Control animals on a daily intake of equivalent to 1 mg of pyridoxine hydrochloric acid had values ranging from 5.0 to 20.8 μg, averaging 11.2. Some of the changes observed in deficient animals are discussed.

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Effect of Enterectomy on Synthesis of Niacin in the Rat.*

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The limited interchangeability of tryptophan and niacin in the nutrition of experimental animals[1] receiving low protein rations has indicated the formation of niacin from this amino acid.[2-4] This synthesis through the intermediate, kynurenine, has been shown to occur in *Neurospora*.[5] Investigations of Ellinger *et al.*,[6] and the marked effect of the character of the carbohydrate on the growth of rats receiving a niacin-low diet containing 9% casein[1] suggested a possible transformation of tryptophan to niacin or its derivatives by intestinal bacteria. The chief arguments against this site of synthesis were the prompt excretion of niacin derivatives in the urine following parenteral administration of tryptophan[7] and more recently the evidence that injection of free tryptophan into the egg results in increased niacin in the chick embryo.[8] A study of the effect of removal of the major portion of the gastro-intestinal tract on the capacity of the animal to form niacin derivatives and excrete them in the urine appeared to be a direct and plausible approach to this problem.

Surgical procedure and ease of catheterization for collection of urine specimens for short periods made the dog preferable to the rat. It was found, however, that the responses to tryptophan in the dog were small compared to those in the rat. N'Methyl nicotinamide was elevated 2-4 fold following the administration of a large dose of tryptophan to the dog, but the acetone-fluorometric method[9] was not suitable in our hands for accurate determination of this metabolite. The amount present was small necessitating the use of large samples, which resulted in high blanks and poor recoveries. Small increases were noted in the nicotinic acid values for dog urine following trytophan administration. The largest increase was noted in the niacin released by acid hydrolysis,[3] but the increase was never more than 100%. Increases up to 100 fold were found in this fraction in rat urine following administration of L-tryptophan either orally or parenterally. The dog appears to have a limited capacity for "converting" tryptophan to niacin derivatives, which is in agreement with the finding that black tongue can be produced with rations containing 19% casein.[10]

Experimental. Male, Sprague-Dawley rats weighing 235-360 g were employed. After receiving stock ration for several weeks, the

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the National Dairy Council, on behalf of the American Dairy Association, to Dr. C. A. Elvehjem, and by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

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1 Krehl, W. A., Sarma, P. S., Teply, L. J., and Elvehjem, C. A., *J. Nutrition*, 1946, **31**, 85.

2 Rosen, F., Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1946, **163**, 343.

3 Singal, S. A., Briggs, A. P., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, 1946, **166**, 573.

4 Schweigert, B. S., Pearson, P. B., and Wilkening, M. C., *Arch. Biochem.*, 1947, **12**, 139.

5 Beadle, G. W., Mitchell, H. K., and Nyc, J. F., *Nat. Acad. Sci.*, 1947, **33**, 155.

6 Ellinger, P., Abdel Kader, M. M., and Emmanuelowa, A., *Brit. J. Exp. Path.*, 1947, **28**, 261.

7 Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, 1947, **168**, 555.

8 Schweigert, B. S., German, H. L., and Garber, M. J., *J. Biol. Chem.*, 1948, **174**, 383.

9 Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1947, **167**, 157.

10 Krehl, W. A., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **158**, 173.

TABLE I.
Effect of Enterectomy on Excretion of Nicotinic Acid and Its Derivatives by Rats Following Tryptophan Administration.

	Urinary excretion, μg per 24 hr per 100 g body weight					
	Free nicotinic acid		Total nicotinic acid following acid hydrolysis		N'methyl nicotinamide	
	Avg	Range	Avg	Range	Avg	Range
Enterectomized rats						
A. Amigen	3.7	1.08- 8.1	8.1	2.24- 14.7	110	19- 240
B. Amigen + *l*-tryptophan	27	5.9 - 91	390	55 - 620	1300	250-3800
Intact rats						
C. Amigen	7.5	7.3 - 7.8	15.1	12.7 - 16.7	120	41- 270
D. Amigen + *l*-tryptophan	71	42 -100	1570	1270 -2020	1090	300-1700

rats were fasted for 24 hours before operating. The gastro-intestinal tracts from the duodenum to the anus were removed from half of the animals, and mock surgery was performed on the remainder. Morphine and light ether anaesthesia were used. After ligating and severing the superior and inferior mesenteric arteries, ligatures were placed on the small intestine, one approximately four inches from the pylorus, just below the pancreas, and another as near the rectum as possible. The gastrointestinal tract between these ligatures was removed by sectioning the gut and all attachments. Animals so treated lived from one to 4 days, when nourished by subcutaneous alimentation as described below. Secretory accumulations were removed from the stomach at 4 hour intervals with a catheter tube and syringe.

The mock surgery on the control animals resembled the enterectomy in that the viscera were handled in the same manner and then replaced intact. The medial incisions were closed with gut sutures for the muscle wall and silk thread for the skin.

The rats were then divided into 4 groups as follows: A. Enterectomized control (4 animals), B. Enterectomized plus added tryptophan (5 animals), C. Intact control (3 animals), and D. Intact animals receiving added tryptophan (3 animals). Within 1-3 hours after surgery the rats were placed in individual metabolism cages, and each received 5 ml of a sterile, modified Amigen.‡ This subcutaneous alimentation was repeated at 4 hour intervals during the entire urine collection period. The control animals in Group A and C received Amigen supplemented with 0.3% sucrose and 0.3% sodium chloride. Groups B and D received the same preparation containing 10 mg of L-tryptophan per ml. During the 24 hour period a total of 6 injections were given for a total dosage of 30 ml for each rat. Autopsy following the experiment showed that the solution was completely absorbed from beneath the skin of the back and neck of each animal.

At the end of the 24 hour collection period the urines were made to volume, filtered, and stored at 5°C under toluene until analyzed. N'Methyl nicotinamide was determined by the acetone-fluorometric method.[9] While this method gave reproducible results in previous studies with urine from normal rats, the results were more variable with these urines, particularly when large quantities of tryptophan were given.

Another portion of each urine sample was neutralized, diluted and analyzed for nicotinic acid by the microbiological assay.[11] The value obtained was termed "free" niacin. A third portion of the urine was hydrolyzed by autoclaving for one hour at 15 lb pressure with an equal volume of 2 N HCl. After neutralizing, "total" niacin was determined microbiologically.[11] The identity and signifi-

‡ Mead Johnson preparation containing 5% enzymatic digest of casein and 5% glucose.

[11] Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. Eng. Chem., Anal. Ed.*, 1943, **15**, 471.

cance of the substance(s) which gives rise to niacin activity for *L. arabinosus* during acid hydrolysis is unknown.[8] The activity of a number of niacin related compounds for *L. arabinosus* have been reviewed by Snell.[12]

Results and discussion. The N′methyl nicotinamide content of the urines (Table I), though variable, was approximately 10 times as high for the rats receiving tryptophan. Enterectomy had no significant effect on this response to tryptophan administration. The average daily excretion of "free" niacin was also increased following tryptophan administration from 3.7 to 27 μg per 100 g of body weight for the enterectomized rats and from 7.5 to 71 μg for the intact animals. Except for one animal whose "free" niacin excretion was 91 μg all of the rats in Group B excreted less than 17 μg, while the intact animals in Group D excreted 42 to 100 μg.

The "total" niacin values increased 100 fold in the intact animals and approximately 50 fold in the enterectomized rats. It should be pointed out that the Amigen administered contains a small amount of tryptophan. The added L-tryptophan, however, resulted in a 15 to 20 fold increase in the total tryptophan intake. At the low tryptophan level (Groups A and C) the N′methyl nicotinamide excretion was much greater than total nicotinic acid. The effect of massive doses of tryptophan was most marked in the latter fraction, resulting in a greater excretion of "total" niacin than N′methyl nicotinamide in Group D. Less than 1% of the administered tryptophan appeared in the urine as the end products measured.

It is evident that the increase in "free" and "total" niacin in the urine following tryptophan administration is somewhat less marked in the enterectomized animals than in intact controls. Whether this difference is a result of the action of bacteria, possibly on tryptophan which has passed into the gut, or of removal of intestinal tissue which participates in the formation of these metabolites, or of the effect of surgical trauma on the metabolic activity of other tissues is not known. It is evident, however, that significant though slightly limited formation of nicotinic acid end products occurred in the enterectomized animals. It seems unlikely that the microbial population in the stomach and a few inches of the intestine could contribute much to urinary nicotinic acid derivatives. Some growth did occur, however, since the stomach contents had a pH of 8-8.5, and there was evidence of putrefactive changes after 2-3 days. It is interesting that enterectomy did not affect the N′methyl nicotinamide excretion, since it has been found to be synthesized in the liver of the rat.[13] The data presented suggest that the tissues, and not the intestinal microflora are the primary sites of formation of these compounds in response to the administration of large amounts of tryptophan. The possibility of the formation of small, though physiologically significant, amounts of niacin derivatives in the tract has not been eliminated.

Summary. 1. The effect of removal of the intestinal tract below the pancreas on the ability of the rat to form niacin derivatives in response to tryptophan administered subcutaneously has been determined.

2. The response in urinary N′methyl nicotinamide was not affected by enterectomy.

3. "Free" and "total" urinary niacin as measured by microbiological assay before and after acid hydrolysis respectively were increased in enterectomized animals although the increase was not as great as in intact animals.

4. The results suggest that the tissues are the primary site of the formation of niacin derivatives in the rat in response to large doses of tryptophan.

[12] Snell, E. E., *Biological Symposia,* 1947, **12**, 183.

[13] Perlzweig, W. A., Bernheim, M. L. C., and Bernheim, F., *J. Biol. Chem.*, 1943, **150**, 401.

16813

Mucolytic Enzyme Systems. V. Anaphylactic Effects on Hyaluronidase Inhibitor in Serum of Normal and Herpetic Rabbits.*

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From the Departments of Physiological Chemistry and Anatomy, University of Minnesota Medical School, Minneapolis.

In an earlier study,[1] it was demonstrated that poliomyelitis infections provoked a significant elevation in the concentration of hyaluronidase inhibitor in blood serum. Good and Campbell[2] showed that anaphylactic shock can precipitate an active encephalitis in rabbits in which *Herpes simplex* virus infection is latent after recovery from an attack of the disease. Accordingly, an investigation was undertaken to determine (1) the effect of *Herpes* infection on the level of the hyaluronidase inhibitor in the blood serum of rabbits,

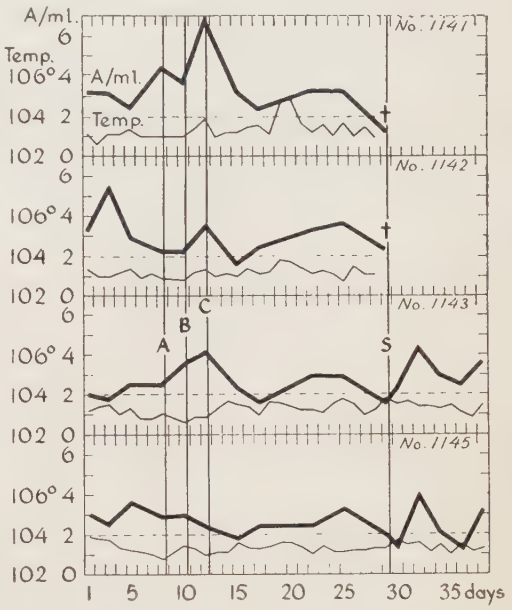

Fig. 1.

Effect of sensitization to egg white, and shock, on the level of hyaluronidase inhibitor in rabbit serum. A, B, C represent the time of sensitizing injections and S the time of the shock injection.

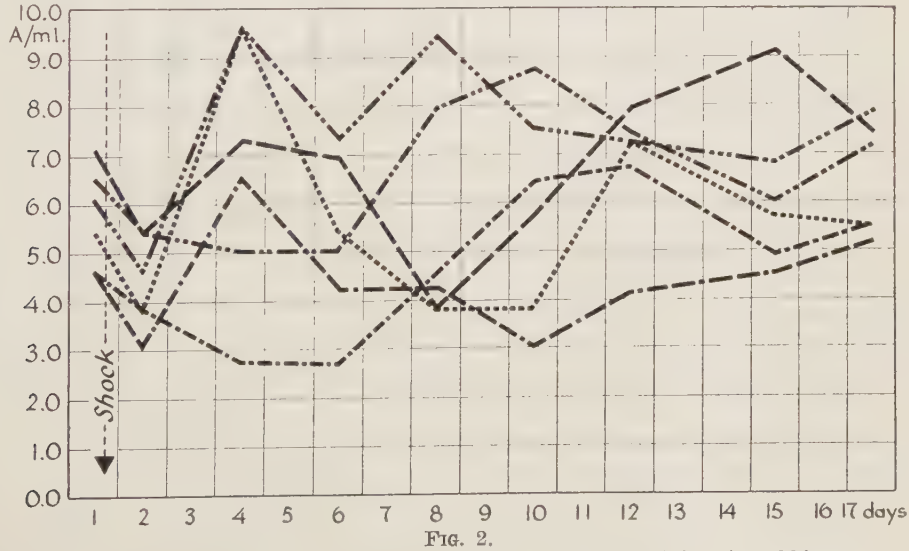

Fig. 2.

Effect of anaphylactic shock on the level of hyaluronidase inhibitor in rabbit serum.

* Supported by grants from the National Foundation for Infantile Paralysis, Inc., and the Division of Research Grants and Fellowships, National Institute of Health, U. S. Public Health Service, Bethesda, Md.

† The authors are grateful for the assistance rendered by Dr. R. A. Good, and the technical aid afforded by Mr. P. Edmondson and Mrs. W. Olsen.

1 Glick, D., and Gollan, F., *J. Inf. Dis.*, 1948, **83**, 200.

2 Good, R. A., and Campbell, B., Proc. Soc. Exp. Biol. and Med., 1948, **68**, 82.

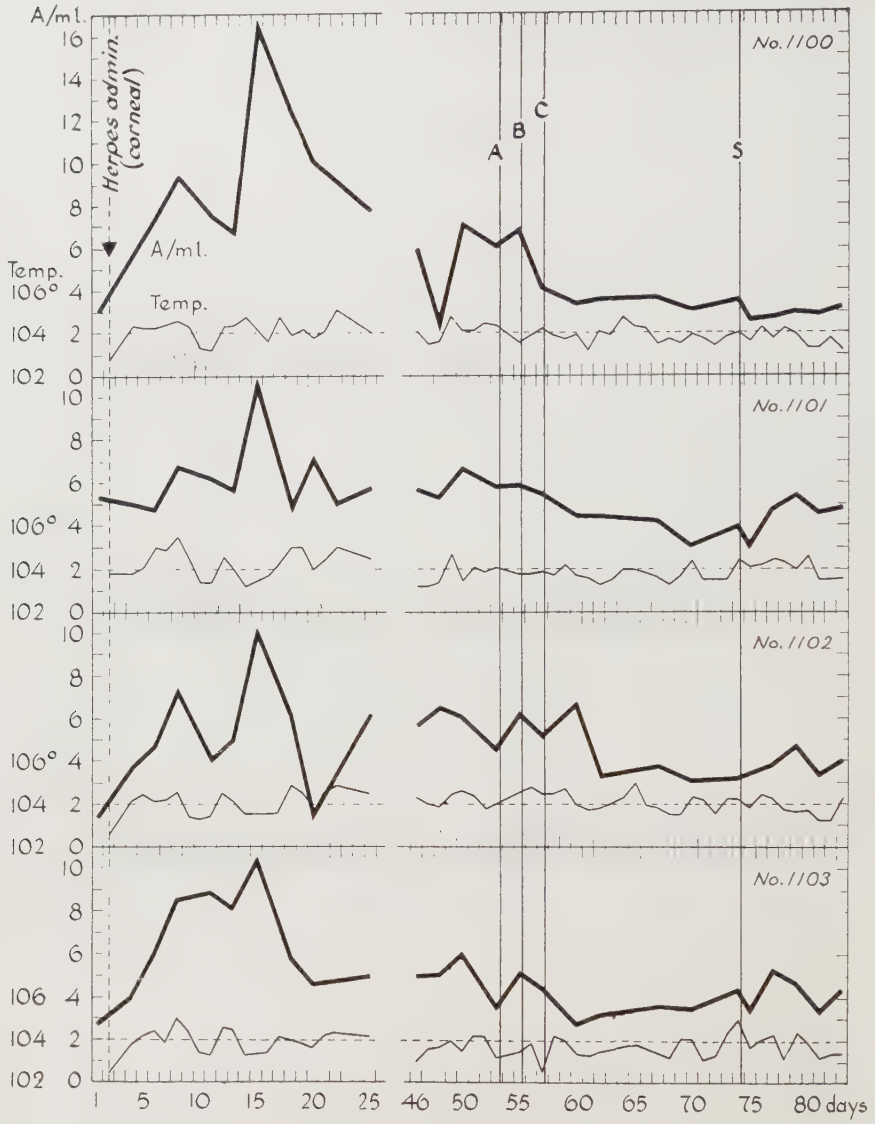

FIG. 3.

Effect of herpetic infection via the cornea, and subsequent sensitization to egg white and shock on the level of hyaluronidase inhibitor in rabbit serum. A, B, C represent the time of sensitizing injections and S the time of the shock injection.

(2) whether a precipitation of active encephalitis by anaphylaxis will be accompanied by a change in the level of the inhibitor, and (3) if an alteration in the latter will result from anaphylaxis *per se*.

Experimental. The preparation of hyaluronic acid from human umbilical cords and hyaluronidase from bull testes, and the viscosimetric measurement of hyaluronidase inhibition by serum were carried out as previously described.[1] The term employed to express the inhibiting property of the serum is $\frac{R - R_o}{R_o}$, designated as (A), where (R_o) represents the time in seconds for the viscosity of the reaction mixture to fall to one-half its initial value, and (R) is the corresponding term for the fall in viscosity in the presence of serum.

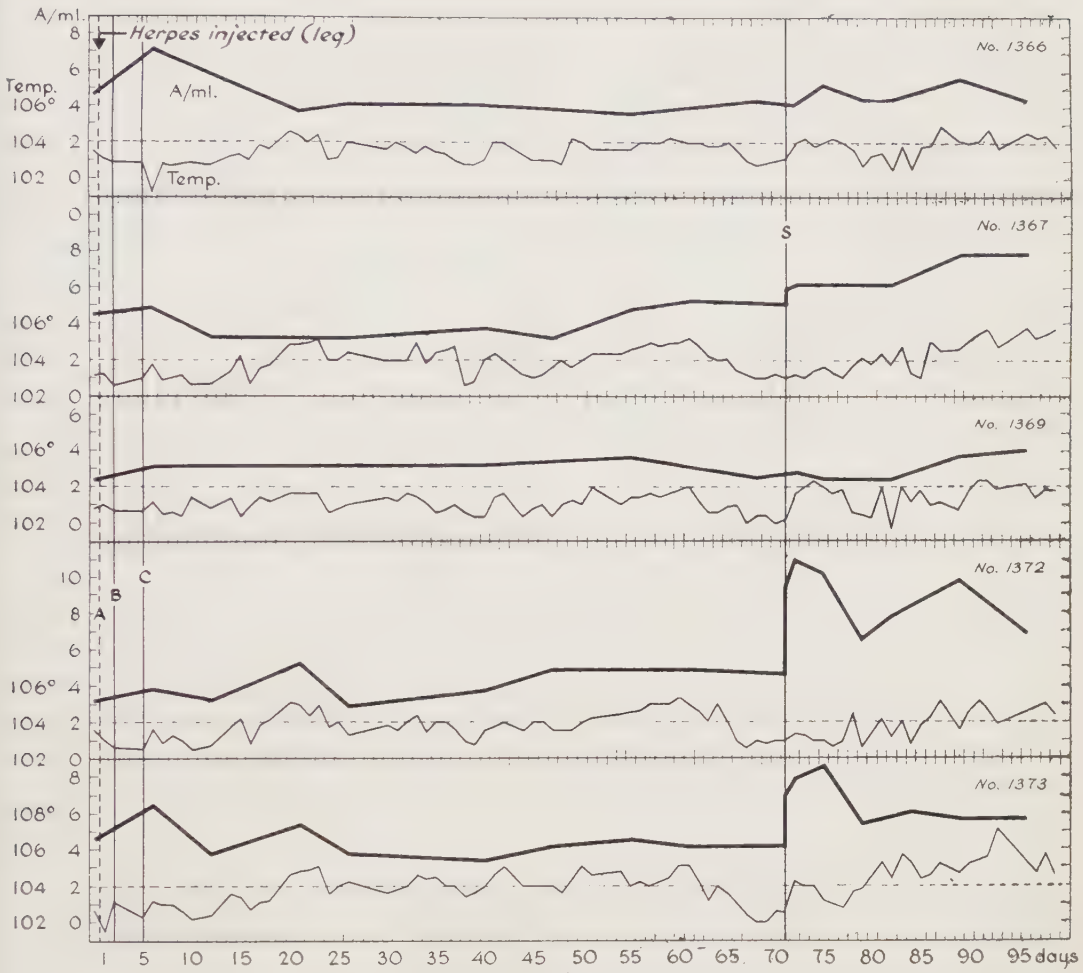

FIG. 4.

Effect of herpetic infection via the leg and subsequent anaphylactic shock on the level of hyaluronidase inhibitor in rabbit serum. A, B, C represent the time of sensitizing injections and S the time of the shock injection.

In the procedure employed, 0.05 cc of serum was required for each determination. The anaphylaxis was effected by sensitizing the rabbits to injections as follows: 1 cc i.v., first day; 0.5 cc i.v., third day; 1 cc i.m., fifth day; and shocking with 0.6 cc i.v. Infections were induced through both the corneal and leg routes. The former were effected by flooding the scarified cornea with a 10% suspension of herpetic mouse brain in physiological saline solution, and the latter by injection of 0.5 cc of the brain suspension into the quadriceps muscle of the right leg.

Results and discussion. The effect of anaphylaxis *per se* on the hyaluronidase inhibitor in blood serum is shown in Fig. 1. The variations which occurred before and during the period of sensitization to the egg white followed no consistent pattern and were of small magnitude. Shock killed 2 of the animals, and in the two that survived, a small elevation in the inhibitor concentration was produced which cannot be considered significant. No consistent effect was observed as a consequence of shocking 6 additional animals. Fig. 2. A small depression was observed the second day after shock, but the magnitude of this effect was slight, and the subsequent changes were widely variable.

Infection through the corneal route pro-

duces an acute systemic reaction in the rabbit which can be followed by means of the animal's temperature. Temperatures exceeding 104° are considered definitely pathognomonic. In animals which survive the disease it is usually difficult to precipitate it again, or as severely, by shock, compared to those which survive after infection through the leg route. On the other hand, the latter group do not usually develop the acute symptoms and high fever that characterize the former during the initial sickness.

These influences are reflected in the changes in the inhibitor concentration. Thus, in Fig. 3, the effect of the corneal infection can be seen to produce a consistent elevation which has a characteristic double peak during the period of acute illness. After recovery, sensitization and shock failed to cause important changes in either the infective state or the inhibitor level.

When the virus was injected into the leg, no large increases in the inhibitor concentration were found, Fig. 4, and the animals were not acutely ill. The sensitization was begun on the same day that the rabbits were infected in order to save time, as these experiments continue for months. From Figs. 1 and 3, it is apparent that sensitization *per se* has no appreciable influence on the inhibitor level. A prompt elevation of the inhibitor level followed shock in those animals in which precipitation of the disease occurred. Animals No. 1372, 1373 showed the most severe symptoms, No. 1366 little effect, and No. 1369 none. The changes in the inhibitor show a correlation with these symptomatic effects.

Summary. Hyaluronidase inhibitor in the blood serum of the rabbit was found to undergo no consistent change in concentration as the result of anaphylaxis induced by egg white. An elevation of the inhibitor level was observed during the acute phase of infection by *Herpes simplex* virus administered via the corneal route. Anaphylactic shock in rabbits which had recovered from the herpetic infection, administered via the leg route, resulted in elevated inhibitor values which were correlated with the severity of the disease thus precipitated from the latent state by the shock.

16814

Effects of Heterologous Seminal Plasma and Sperm Cells on Fertilizing Capacity of Rabbit Spermatozoa.

M. C. CHANG. (Introduced by D. Rapport.)

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In a previous experiment,[1] the beneficial effects of rabbit seminal plasma on the fertilizing capacity of rabbit spermatozoa was demonstrated. This paper reports the effects of human, bull, and rabbit seminal plasma, as well as of dead sperm of these species, on the fertilizing capacity of rabbit spermatozoa.

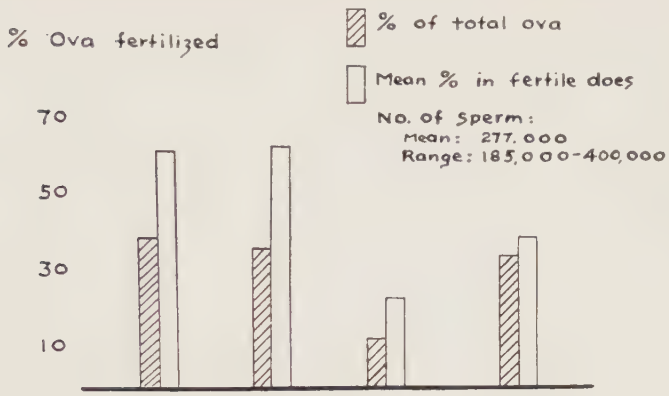
Methods: Human semen was collected into a sterile tube and stored at 2°C no more than 12 hours. Bull semen and vasectomized rabbit semen were obtained by means of an artificial vagina[2,3] about 1 to 2 hours before use. Human and bull samples were centrifuged at 2,000 R.P.M. for about 30 to 40 minutes and the supernatant fluid was used as seminal plasma.

The semen of a single rabbit, collected at

1 Chang, M. C., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 51.

2 Walton, A., Notes on Artificial Insemination of Sheep, Cattle and Horses. 1942, London: Holborn Instrument Co.

3 Macirone, C., and Walton, A., *J. Agri. Sci.*, 1938, **28**, 122.



Treatment	Human Sem. plasma	Rabbit Sem. plasma	Bull Sem. plasma	Fructose Ringer Solution
No. of does	16	14	14	16
Sterile does	7	4	7	4
Total ova	498	412	517	507

FIG. 1.
Effects of heterologous seminal plasma on fertilizing capacity of rabbit spermatozoa (immediate insemination).

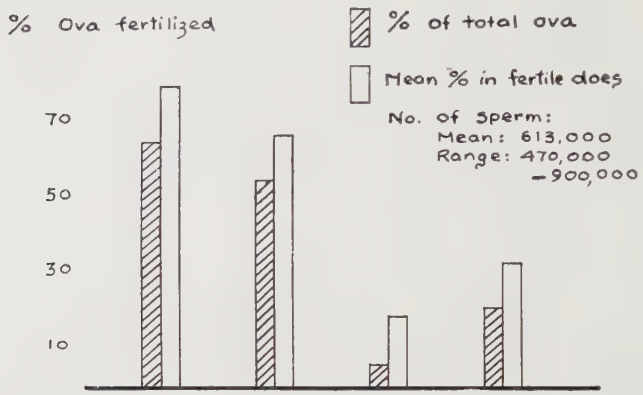
intervals of 7 days, was used throughout this investigation. About 0.01 to 0.03 ml of semen, depending on the concentration of sperm, was first suspended in 10 ml of Fructose Ringer solution (NaCl, 0.85 mg; KCl, 0.042 g; $CaCl_2$, 0.024 mg; $MgCl_2$, 0.02 g; $NaHCO_3$, 0.1 g; Fructose, 0.1 g; Double glass distilled water, 100 ml). The sperm concentration of this suspension was immediately and quickly examined by means of a haemocytometer technic in order to obtain a minimal effective number of sperm (Number of spermatozoa required to fertilize only a portion of the ova ovulated). Then 0.5 ml of this suspension was added to 0.5 ml of human, bull, rabbit seminal plasma, or to 0.5 ml of Fructose Ringer solution which was used as a control. The mixture (all 1 ml in volume) was inseminated, immediately in the first series of experiments, or after storage at room temperature for one hour in the second series of experiments. After insemination, the number of spermatozoa was counted again 4 to 6 times to obtain an accurate number of spermatozoa inseminated.

Six to 8 doe rabbits (superovulated according to Pincus[4]) were used at a time. Two does each were inseminated with the same sperm mixture. The ovulation injection of pituitary extract was given just after insemination. The animals were sacrificed about 25 hours later. The ova of each rabbit were flushed from fallopian tubes with undiluted rabbit serum and the fertilized and unfertilized ova were counted.

In a third series of experiments, sperm cells of human, bull or rabbit were obtained by centrifugation of semen samples and then resuspended in saline and centrifuged again. The washed sperm cells were deep frozen with a mixture containing solid carbon dioxide and acetone and then suspended in Fructose Ringer solution (about 800 millions of dead sperm per ml). A minimal effective number of rabbit spermatozoa was suspended in this fluid and stored at room temperature for one hour before insemination. In this series, one part of fresh egg yolk was suspended into one part of Fructose Ringer solution as a control.

Results: Fig. 1-3 illustrate the results. The fertilizing capacity of spermatozoa under different treatments was expressed in terms of percentage of ova fertilized. A few of the does (31%) had no ova fertilized, due to female infertility and/or to the small number

[4] Pincus, G., *Anat. Rec.*, 1940, **77**, 1.



Treatment	Human Sem. plasma	Rabbit Sem. plasma	Bull Sem plasma	Fructose Ringer Solution
No. of does	8	8	8	8
Sterile does	1	1	3	5
Total ova	179	191	372	239

FIG. 2.

Effects of heterologous seminal plasma on fertilizing capacity of rabbit spermatozoa (stored one hour before insemination).

Treatment	Human Sperm	Rabbit Sperm	Bull Sperm	Egg yolk
No. of does	6	10	8	10
Sterile does	0	3	1	3
Total ova	145	273	208	303

FIG. 3.

Effects of dead sperm of different species on fertilizing capacity of rabbit spermatozoa (stored for one hour before insemination).

of sperm inseminated. Thus, the percentages of all ova, including the ova of sterile does, in each group and the mean percentage of ova fertilized in the fertile does were presented in figures. In the calculation of statistical significance between each group, the mean percentages of fertilized ova of all does, including sterile does, was taken.

Fig. 1 illustrates the results of 60 inseminations performed immediately after prepara-

tion of the mixture. The percentage of ova fertilized was low when rabbit sperm were suspended in bull seminal plasma or in Fructose Ringer solution. Statistical analysis of the data (by t test) however, shows that there is no significant difference between human and rabbit seminal plasma and Fructose Ringer solution, but there is a significant difference between bull seminal plasma and other sperm mixtures ($t = 2.3$, 2.78, or 2.4, $p < .05 > .01$). This clearly demonstrates that bull seminal plasma has an adverse effect on the fertilizing capacity of rabbit sperm.

Fig. 2 illustrates the results of another 32 inseminations performed when the sperm mixtures were allowed to stand for one hour before insemination. The beneficial effects of human or rabbit seminal plasma as compared with Fructose Ringer solution or bull seminal plasma is clearly shown. There is a very significant difference between human and bull seminal plasma ($t = 3.9$, $p < .01$), human and Ringer solution ($t = 3.55$, $p < .01$), rabbit and bull plasma ($t = 3.75$, $p < .01$) and rabbit and Ringer solution ($t = 3.12$, $p < .01$).

It is clear by comparison of Fig. 1 and 2, that human and rabbit seminal plasma have a beneficial effect, while bull seminal plasma has an immediate harmful effect, on the fertilizing capacity of rabbit spermatozoa. Fructose Ringer solution has no immediate ill effect, but the fertilizing capacity of rabbit spermatozoa decreases after one hour in Fructose Ringer solution.

Fig. 3 illustrates the result of another 34 inseminations when the minimal effective number of rabbit spermatozoa were suspended in Fructose Ringer solution containing dead human, bull and rabbit sperm cells or egg yolk. The percentage of ova fertilized was high when rabbit spermatozoa were mixed with human or bull sperm cells, but it was rather low when mixed with rabbit sperm cells or with egg yolk. There is a significant difference between human sperm cells and egg yolk ($t = 2.8$, $p < .02 > .01$); the difference between other treatments is not significant. Since egg yolk is considered the best medium for the preservation of fertilizing capacity of spermatozoa in the practice of artificial insemination,[5] it is of interest to note that sperm cells of human and bull are better than or equal to egg yolk.

The results presented in Fig. 2 and 3 are comparable because the sperm mixtures were stored for one hour before insemination. In this respect, human, rabbit, or bull sperm cells and human or rabbit seminal plasma are beneficial to the fertilizing capacity of rabbit spermatozoa (No statistical significant difference) while bull sperm cells are much better than bull seminal plasma (55% with significant difference). Fructose Ringer solution with egg yolk is definitely better for the preservation of fertilizing capacity than without egg yolk (28%, with significant difference). Human sperm cells are better than Fructose Ringer solution containing egg yolk (39%, with significant difference).

Discussion. In the previous experiment,[1] the beneficial effect of seminal plasma was demonstrated when 0.9% NaCl was used as control. It was thought that 0.9% of NaCl may have an adverse effect on spermatozoa. The present study once again demonstrated the same fact when a balanced salt solution with the metabolic substrate, fructose[6] was used.

In a study of the effects of high dilution on fertilizing capacity[7] Chang has postulated that there might be a beneficial substance in the seminal plasma or in the spermatozoa. The present study clearly verifies this assumption. Whether the beneficial effect of seminal plasma or egg yolk is to prevent the escape of essential substances in the spermatozoa as suggested by Emmens and Swyer[8] or to contribute an essential substance for the prolongation of fertilizing capacity is hard to say at present. If sperm cells, seminal plasma or egg yolk contribute this essential substance, the sources of this substance may be widely distributed in animal tissues. Consid-

5 Anderson, J., The Semen of Animals and Its Use in Artificial Insemination. 1947: Edinburgh, Genetic Institute.

6 Mann, T., *Nature*, 1946, **157**, 79.

7 Chang, M. C., *Science*, 1946, **104**, 361.

8 Emmens, C. W., and Swyer, G. I. M., *Nature*, 1947, **160**, 718.

ering the beneficial effect of bull sperm cells on rabbit spermatozoa and the harmful effect of bull seminal plasma, the presence of this essential substance in the spermatozoa of different species rather than in the seminal plasma is indicated.

It is of biological interest to note the compatibility of rabbit sperm and human seminal plasma and the incompatibility of rabbit sperm and bull seminal plasma.

A high dilution of sperm was employed in the present study in order to obtain a minimal effective number of spermatozoa. It was observed that the progressive movement of sperm lasted for 5 to 6 hours in seminal plasma (only 3 to 4 hours in bull seminal plasma) or in the solutions containing added dead sperm. But only sluggish movement was observed in Fructose Ringer solution from the beginning for 2 to 3 hours.

Summary. Superovulated does were inseminated with a minimal effective number of sperm suspended in Fructose Ringer, or seminal plasma of human, bull or rabbit; or in Fructose Ringer suspension of egg yolk, or deep frozen sperm. It was found that the seminal plasma of human or rabbit had a beneficial effect while that of bull had an ill effect on the fertilizing capacity of rabbit sperm as compared with Fructose Ringer. Fructose Ringer containing dead sperm of human, rabbit or bull was found as good or better media for the preservation of sperm as egg yolk; which indicates the presence of essential substances in the sperm cells for the maintenance of their fertilizing capacity.

The writer wishes to acknowledge his gratitude to Dr. G. Pincus for encouragement, to Mr. C. Putney of Massachusetts Selective Breeding Association for supplying bull semen, to Mr. R. Gunnarson for assistance, to the Foundation for Applied Research, San Antonio, Texas, and the Committee of Human Reproduction of National Research Council for Grant in Aid.

16815 P

Occurrence of Streptomycin Resistant Tubercle Bacilli in Mice Treated with Streptomycin.*

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Tubercle bacilli readily become resistant to streptomycin *in vitro*[1-3] and in patients being treated with streptomycin.[1,4-6] Feldman, Karlson and Hinshaw[7] reported the isolation of streptomycin resistant tubercle bacilli from 3 of 8 guinea pigs treated for a prolonged period with 6.0 mg streptomycin per day, after these had been infected with a streptomycin sensitive strain of *M. tuberculosis*.

Methods. One hundred mice were infected intravenously with 1.0 mg of a suspension of a culture of *M. tuberculosis* var. hominis (strain no. 24)[3] which we had previously

* This investigation was supported (in part) by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

1 Youmans, G. P., Williston, E. H., Feldman, W. H., and Hinshaw, H. C., *Proc. Staff Meetings, Mayo Clinic,* 1946, **21**, 126.

2 Middlebrook, G., and Yegian, D., *Am. Rev. Tuberc.*, 1946, **54**, 553.

3 Williston, E. H., and Youmans, G. P., *Am. Rev. Tuberc.*, 1947, **55**, 536.

4 Youmans, G. P., and Karlson, A. G., *Am. Rev. Tuberc.*, 1947, **55**, 529.

5 Streptomycin Committee, Veterans Administration, Report to the Council on Phar. and Chem., *J.A.M.A.*, 1947, **135**, 634.

6 Streptomycin Committee, Veterans Administration, Report to the Council on Phar. and Chem., *J.A.M.A.*, 1948, **138**, 584.

7 Feldman, W. H., Karlson, A. G., and Hinshaw, H. C., *Am. Rev. Tuberc.*, 1948, **57**, 162.

TABLE I.
Occurrence of Streptomycn-Resistant Tubercle Bacilli in Mice Treated with Streptomycin.

No. of mice	Amt streptomycin sulphate in mg per day	% mortality	Avg survival time in days*	No. of mice which developed streptomycin resistant strains			Range of sensitivity to streptomycin sulphate† (in mg per ml of medium)
				Total %	Sacrificed %	Died %	
19	1500	26.3	131.6	17 (89.4)	14 (100)	3 (60)	12.5 to >1000
20	750	85	73.5	13 (65.0)	3 (100)	10 (58.8)	12.5 to >1000
18	375	100	40.1	4 (22.2)	0	4 (22.2)	12.5 to 500
18	187.5	100	19.7	1 (5.5)	0	1 (5.5)	12.5
20	0	100	14.6	0	0	0	0.19 to 1.56

* Includes mice sacrificed at 155 days.

† Indicates difference in sensitivity to streptomycin of cultures from individual mice. No significant difference in range of sensitivity was noted between mice sacrificed and those that died.

shown readily became resistant *in vitro*.[3] This culture, however, was sensitive *in vitro* to between 0.19 and 1.56 μg per ml of streptomycin sulphate.[3] The mice were divided into groups and treated by subcutaneous injection twice daily with one-half the amounts of streptomycin shown in Table I. Experimental attrition reduced the number of mice on which complete data were obtained to 95. Treatment was continued for a period of 155 days, at the end of which time, surviving mice were sacrificed. At the time each mouse died, or was sacrificed, it was autopsied, and one lung fixed in 3.7% formaldehyde, and gross and microscopic examination made as previously described.[8-11] Isolation of tubercle bacilli was accomplished by grinding the other lung in a sterile mortar, and treating the residue with oxalic acid, as described by Corper and Uyei,[12] and planting on Herrold's medium.[13] When sufficient growth was obtained, these cultures were tested in serum synthetic liquid media for their sensitivity to streptomycin.

Results. Table I shows the results obtained. It is readily apparent that a large proportion of the mice developed streptomycin resistant tubercle bacilli. The incidence of the occurence of streptomycin resistant strains of tubercle bacilli in the different groups of mice was directly related to the amount of streptomycin administered. A similar relationship existed between the development of resistance and the average survival time. The two smaller doses of streptomycin apparently were not sufficiently large to suppress the infection for a period which would permit the multiplication of the resistant organisms to a detectable number.

Results essentially similar to these have also been obtained with H37Rv strain of *M. tuberculosis*.

In many of the mice, the pathological findings confirmed the bacteriological evidence of the presence of streptomycin resistant tubercle bacilli. Evidence of active necrotic disease superimposed upon healed or healing areas was common in many of the animals from which streptomycin resistant tubercle bacilli were isolated.

Since we have found that streptomycin resistant tubercle bacilli appear in a large proportion of mice treated with adequate amounts of streptomycin, an experimental method is now available for the determination of the effect of various factors, including combined therapy, on the development of resistance to streptomycin *in vivo*.

Summary. Streptomycin resistant tubercle bacilli were obtained from the majority of intravenously tuberculized mice treated with amounts of streptomycin which permitted a relatively long survival time.

8 Youmans, G. P., and McCarter, J. C., *Am. Rev. Tuberc.*, 1946, **52**, 432.

9 Raleigh, G. W., and Youmans, G. P., *J. Inf. Dis.*, 1948, **82**, 197.

10 Raleigh, G. W., and Youmans, G. P., *J. Inf. Dis.*, 1948, **82**, 205.

11 Youmans, G. P., and Raleigh, G. W., *J. Inf. Dis.*, 1948, **82**, 221.

12 Corper, H. J., and Uyei, N., *J. Lab. and Clin. Med.*, 1930, **15**, 348.

13 Herrold, R. D., *J. Inf. Dis.*, 1931, **48**, 236.

16816

Effect of X-rays on the Migration of Cells from Adult Tissue Explants.*

R. S. Hoffman and S. H. Wollman. (Introduced by W. deW. Andrus.)

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A considerable literature exists on the effects of irradiating tissue cultures *in vitro* covering studies which have been made on the effect of radiation on mitosis, cell migration, cell morphology and viability.[1,2] In these investigations, generally, active growing cultures of various types of tissues have been used, but no work as far as we know has been done on the effects of radiation on freshly explanted normal adult tissues. When a piece of such normal adult tissue is removed from an animal and cultivated *in vitro,* a period of time called the latent period elapses before any cells appear at the margin of the explant.[3] Our present studies were undertaken to determine the effects of various doses of X-ray on the duration of the latent period.

Experimental method. Adult chicken cardiac muscle was cut into small fragments (approximately 2 mm^3). Groups of from 4 to 9 of these fragments were placed in Carrel flasks containing 0.5 cc of chicken plasma, 1 cc of Tyrode's solution and one drop of embryonic extract to facilitate formation of a coagulum. These flasks were then placed in an incubator at 38°C for a period of 20 minutes, exposed for 5 minutes to 5°C (the short 5°C exposures occurred during transport of cultures), after which they were again kept at 38°C for 20 minutes and then irradiated. During the entire period of irradiation (about 10 minutes) experimental as well as the control flasks were kept at 25°C. Following irradiation, the flasks were again subjected to 5°C for 5 minutes, after which they were kept in the incubator at 38°C. All flasks were removed from the incubator for 15 minutes every 6 hours in order to observe the tissue fragments under the microscope and note the appearance of the first cells. Each curve in Fig. 1 represents the collected observations on 30 to 40 pieces of tissue.

The source of the X-rays was a 180 Kv tungsten target experimental X-ray machine, running at 25 milliamperes and using a filtration of 0.5 mm Cu + 1.65 mm Al; the half value layer of transmitted X-rays was 0.8 mm

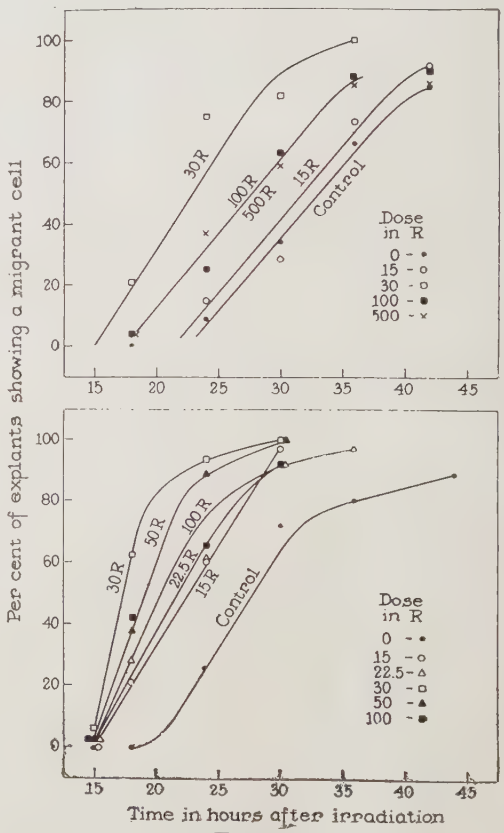

Fig. 1.

Curves showing how migration of cells from explants of adult chicken heart depends on the time after irradiation for various doses of X-rays.

* This work was supported by a grant furnished by the Committee on Growth of the National Research Council.

1 Spear, F. G., *Brit. J. Radiol.,* 1935, **8**, 68, 280.

2 Lea, D. E., Action of Radiations on Living Cells, Pub. Cambridge-Macmillan, 1947.

3 Hoffman, R. S., Goldschmidt, J., and Doljanski, L., *Growth,* 1937, **1**, 228.

Cu. The tissues were at a distance of 50 cm from the target. The studies reported here in detail involved doses of 500 r or less at a dose rate of about 25 r per minute. Some studies were also made using doses up to 10,000 r of unfiltered radiation at a higher dose rate.

Results. Explants given small doses of X-rays had a shorter latent period than unirradiated controls. This seems to be true for doses of X-rays at least as high as 500 r.

Data for two representative experiments are shown in the graphs, Fig. 1, which indicate the percentage of the explants which show one or more migrating cells as a function of the time after irradiation for various doses of X-rays. Typical sigmoid curves are obtained.

In order to compare the effects of different doses of radiation more easily, we have plotted in Fig. 2 data obtained from graphs like those in Fig. 1, giving the time after irradiation at which 50% of the explants show one or more migrating cells as a function of the dose of X-rays for the above experiments. Different heart preparations have different latent periods for both controls and irradiated explants. A fact which is consistent among all preparations, however, is that the minimum latent period occurs at a dose of about 30 r. At higher doses of irradiation the latent period increases slowly with dose and it is not until explants are irradiated with 1,000 r that the latent period is as long as in the controls. With doses of 10,000 r the latent period is approximately 50% longer than in the controls, but the result may not be strictly comparable since the dose rate was used for the 10,000 r is considerably higher than that with smaller doses.

One experiment using 11-day old embryonic chick heart fragments gave results similar to those obtained with adult heart tissue, although as is characteristic of embryonic tissue the latent periods were very short. The latent period was less than 2 hours for 30 r, approximately 3 to 4 hours for controls and 5 hours for 5,000 r.

Our data in Fig. 2 indicate that irradiation has two apparent effects on the duration of the latent period. At doses below 30 r the

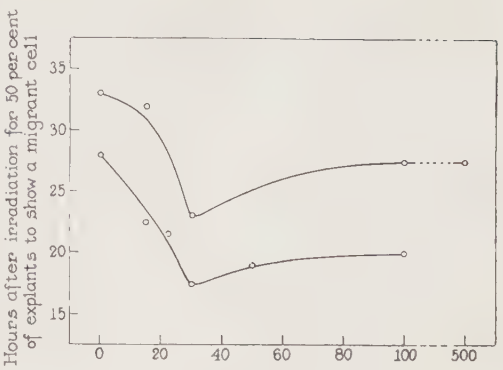

FIG. 2.

Curves showing how the time for 50% of explants to show one or more migrating cells depends on the dose of X-rays expressed in roentgens.

most obvious effect is a stimulating effect on the migration of cells from a fresh explant. With doses of X-rays higher than 30 r the gradually increasing latent period suggests a superimposed inhibition which increases with the dose of radiation. This inhibition of migration with larger doses of X-ray is not surprising since practically all the older literature shows that irradiation of cell cultures causes an inhibition which becomes evident only after a delay of approximately 24 hours. The stimulating effect of small doses of irradiation is somewhat more surprising. Ludford[4] observed in a few cases a stimulating effect of radiation on actively growing cultures but made no systematic and controlled study. He also observed that in growing cultures macrophages seem to inhibit the growth of fibroblasts. In these cultures the growth stimulating effect of irradiation was attributed to the inhibition of the macrophage activity by X-rays. In our experiments on fresh explants, however, the mechanism responsible for the stimulation or inhibition may be different.

Another explanation which has been offered[5] is that radiation damages some cells, causing a release of nutrients which can stimulate other cells into activity. This occurs with considerably larger doses of radiation than those used by us.

Our results may be of interest in connec-

[4] Ludford, R. S., *Proc. Roy. Soc.*, 1934, **115**, 278.

[5] Giese, A. C., *Quart. Rev. Biol.*, 1947, **22**, 253.

tion with the observation of Kaplan[6] who found that irradiation of a lymphosarcoma *in vivo* with 400 r significantly increased the number of metastases over the number occurring in unirradiated controls.

Summary. Evidence is presented that irradiation with small doses of X-rays decreases the time—latent period—required for a migrating cell to appear at the margin of a fresh explant of normal adult chicken heart. The maximum shortening of the latent period occurs with a dose of about 30 r. With larger doses of X-rays the latent period increases again, although a dose of the order of 1,000 r is necessary before the latent period is as long as that of the controls.

[6] Personal communication.

16817

Activity of Vitamin B_{12} in the Growth of Chicks.*

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The requirement of the growing animal for certain dietary essentials can be increased by inducing a hyperthyroid condition. Ershoff,[1] and Betheil *et al.*[2] observed that liver effectively counteracted a thyrotoxicity in rats which were fed desiccated thyroid. Robblee *et al.*[3] found that the addition to the basal ration of either desiccated thyroid or iodinated casein improved the effective assay range for an unidentified chick growth factor. Work has been in progress[4] for some time on the properties of the growth stimulating components of condensed fish solubles. Fractionation procedures indicated similarities between the chick factor and the material in liver which is active in the treatment of pernicious anemia. Injectable liver extracts were found to be active in promoting the growth of chicks fed rations which were complete in the known growth essentials.[5]

Rickes *et al.*[6] reported the isolation from liver of a red crystalline compound termed vitamin B_{12} which was active in microgram quantities for the remission of pernicious anemia in relapse. More recently Ott *et al.*[7] showed that crystalline vitamin B_{12} had "animal protein factor" activity for the chick. Our data confirm this observation and indicate that vitamin B_{12} is highly active in stimulating the growth of the hyperthyroid chick.

Method. The chicks (New Hampshire ♂♂ x Single Comb White Leghorn ♀♀) were the progeny of hens fed diet B_1 described previously[8] in which protein was provided by

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by funds supplied by the Commercial Solvents Corporation, Terre Haute, Ind., and by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

[1] Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 500.

[2] Betheil, J. J., Wiebelhaus, V. D., and Lardy, H. A., *J. Nutrition*, 1947, **34**, 431.

[3] Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 400.

[4] Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *J. Biol. Chem.*, 1948, **173**, 117.

[5] Nichol, C. A., Robblee, A. R., Cravens, W. W., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **170**, 419.

[6] Rickes, E. L., Brink, N. G., Konuiszy, F. R., Wood, T. R., and Folkers, K., *Science*, 1948, **107**, 396.

[7] Ott, W. H., Rickes, E. L., and Wood, T. R., *J. Biol. Chem.*, 1948, **174**, 1047.

[8] Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *Poultry Sci.*, 1948, **27**, 442.

TABLE I.
Response of Chicks to Supplementation with Vitamin B_{12}.

Group No.	Supplement	No. of chicks surviving	G gain during 14-day test period	Hemoglobin, g
1	None	7	105	8.33
2	3% condensed fish solubles	10	190	8.52
3	Vit. B_{12} 0.75 γ/100 g ration	10	152	8.42
4	" " 1.5 γ/100 g "	9	193	8.98
5	Liver extr. (Lilly, reticulogen 20 U.S.P. units/cc), 0.5 U.S.P. unit bird/day*	10	191	8.52
6	Vit. B_{12}, 0.01/γ/bird/day*	10	126	8.84
7	" " 0.1 γ/ " "	10	177	8.81
8	" " 0.5 γ/ " "	10	184	8.60

* Injected intramuscularly.

yellow corn, wheat, soybean oil meal, fish meal and meat scrap. The experimental groups were housed in electrically heated batteries with raised screen floors. Feed and water were supplied *ad libitum*. The basal ration contained ground yellow corn 35, soybean oil meal 28, wheat bran 10, wheat middlings 10, dehydrated alfalfa meal 5, vitamin test casein 7.5, limestone grit 2.0, steamed bonemeal 1.5, iodized salt 0.5, fish oil (2000A-400D) 0.5, $MnSO_4 \cdot H_2O$ 0.025, iodinated casein (Protamone, 3.07% thyroxine) 0.05 g; thiamine 0.3, riboflavin 0.6, niacin 5.0, calcium pantothenate 2.0, pyridoxine 0.4, p-aminobenzoic acid 10, choline chloride 150, inositol 100, folic acid 0.05, biotin 0.02, menadione 0.05, and α-tocopherol 0.3 mg.

The chicks were wing-banded and weighed when one day old and placed on the basal ration. Individual weights were recorded at weekly intervals. Selection of the birds to be used for the experiment was made at the end of a 2 week depletion period. Mortality during the first 2 weeks was 9%. In order to limit individual variation, two criteria for selection were used: (1) weight (chicks below 60 or above 105 were discarded) and (2) rate of gain (the % gain during the second week was calculated for each bird and those within the range of 33 to 59% were kept). Of the chicks surviving the 2 week depletion period 17% were discarded on the basis of weight and 16% were discarded on the basis of % gain. The selected chicks were distributed (10 per group) in such a way that the average weights of the groups fell between 85 and 88 g and the average % gain ranged from 47 to 48%.

Supplements were added on the 15th day and continued for a 14 day period. Supplementation of the basal ration with 3% condensed fish solubles was made at the expense of corn. The vitamin B_{12} solution (25γ/ml) supplied by Dr. D. F. Green of Merck and Company, Inc., Rahway, N. J., was diluted with distilled water and thoroughly mixed into the basal ration by a mechanical mixer to provide 0.75 γ and 1.5 γ of vitamin B_{12} per 100 g. Injections of vitamin B_{12} and liver extract were made into the pectoral muscle using a 1 cc tuberculin hypodermic syringe calibrated in 0.01 cc. Dilutions of vitamin B_{12} with distilled water were such that 0.2 cc injected every other day provided the dosages listed in Table I. Hemoglobin determinations were made on all chicks on the final day of the test period.

Results. The results are presented in Table I. The positive control for groups given vitamin B_{12} in the basal ration was a group receiving a supplement of 3% condensed fish solubles. Groups receiving vitamin B_{12} by injection were compared with a group injected with a liver extract (Lilly, reticulogen, 20 U.S.P. units per cc), at a dosage of 0.5 U.S.P. units per bird per day. The two positive controls showed average weight differences from the negative control of 85 g (condensed fish solubles) and 86 g (reticulogen).

Pure vitamin B_{12} at a level of 0.75 γ per 100 g of ration caused an average increase in weight of 47 g over the unsupplemented group. At a level of 1.5 γ of vitamin B_{12} per 100 g of ration the average weight in-

crease was 88 g more than the negative control.

Vitamin B_{12} was injected at 3 dosage levels: 0.01 γ, 0.1 γ and 0.5 γ per bird per day. The growth response was 21 g at the 0.01 γ level, 72 g at the 0.1 γ level and 79 g at the 0.5 γ level.

No significant difference was noted in the average hemoglobin values of the experimental groups.

Discussion. It is apparent that pure vitamin B_{12} is able to replace the growth stimulating properties of condensed fish solubles or injectable liver extract under the experimental conditions described. It is a reasonable assumption that the properties of these complex materials which have been under study by means of fractionation procedures and chick growth assay, are due to the occurrence of vitamin B_{12}.

The thyrotoxic condition in chicks induced by feeding a ration containing 0.05% iodinated casein is characterized by the hyperexcitability of the birds and depressed rate of gain in weight. The growth response to supplementation is not dependent upon a hyperthyroid condition but the use of a basal ration containing iodinated casein increases the magnitude of the growth response.[3] The role of vitamin B_{12} in counteracting a thyrotoxic condition will require further detailed investigation.

When vitamin B_{12} was fed at a level of 0.75 γ per 100 g of ration the growth response was highly significant and the rate of gain was approximately half maximal under the conditions of this assay. At a level of 1.5 γ vitamin B_{12} per 100 g of ration the growth response compared closely with that of a group receiving 3% condensed fish solubles. This material is recognized as a potent source of the animal protein factor and the rate of gain induced by a 3% level of condensed fish solubles was observed in previous studies to be close to maximum.

Injection of vitamin B_{12} at a level of 0.01 γ per bird per day resulted in a marginal growth response. At a dosage of 0.5 γ per bird per day the response compared closely with that of a group injected with liver extract (Lilly, reticulogen) at a level of 0.5 U.S.P. unit per bird per day. This liver extract was previously observed to be highly effective in stimulating the growth of the hyperthyroid chick.[5] The response observed at a level of 0.1 γ vitamin B_{12} per bird per day was not appreciably lower that that at the 0.5 γ level.

Summary. Vitamin B_{12} administered orally or parenterally completely counteracted a thyrotoxic condition in chicks produced by feeding a basal ration containing 0.05% iodinated casein. A level of 0.75 γ vitamin B_{12} per 100 g of ration resulted in a half maximal growth response. At a level of 1.5 γ vitamin B_{12} per 100 g of ration the growth response after a two week test period was 88 g compared to 86 g for a group supplemented with 3% condensed fish solubles. A response of 72 g resulted from the intramuscular injection of 0.1 γ vitamin B_{12} per bird per day.

Pure vitamin B_{12} can replace the animal protein factor activity of condensed fish solubles and injectable liver preparations.

We are indebted to Merck and Co., Inc., Rahway, N. J., for pure vitamin B_{12} and for crystalline vitamins; to the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y., for synthetic folic acid; to the Cerophyl Laboratories, Inc., Kansas City, Mo., for protamone; and to the Borden Company, New York, for condensed fish solubles.

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Lipotropic Activity of Various Compounds Under Standardized Conditions.

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A large number of compounds has been tested for lipotropic activity since the demonstration of the action of choline on liver fat.[1] The laboratories carrying out such studies have used various experimental conditions for demonstrating lipotropic activity. It has often been suggested that the lack of uniformity in conditions has hindered correlation of the data obtained by different investigators. In testing the activity of various substances mice,[2] rats,[3] and depancreatized dogs[4] have been used. The stage of the life cycle of the experimental animal has received little consideration. The diets used for producing fatty infiltration of the liver have ranged from essentially fat-free to very high-fat diets, from protein-free to approximately 25% protein diets.[1] The carbohydrate component has been varied in amount and kind, and dietary mixtures of 2 or 3 different proteins have been utilized.[1] Incomplete vitamin mixtures have been used in special cases.[1] In view of the varying conditions under which different compounds have been reported to be lipotropically active, it seemed important to study a representative group of these compounds under standardized conditions. The substances chosen for study have previously[1] been shown to be lipotropically active.

Experimental. The experimental conditions and techniques were those used in this laboratory in previous studies of lipotropism.[5] Male rats of the Carworth strain weighing approximately 170 g were used. The basal diet contained 15.4% casein, 3.2 % arachin, 5% salt mixture, 2% cellu flour, 34.4% glucose, and 40% lard. This purified choline-free diet has been used extensively in this laboratory in studies of growth and lipotropism. It supplies 500 mg of methionine and 100 mg of cystine per 100 g of diet. Animals of the Carworth strain and of this body weight grew at a slightly slower rate on the basal diet than the strain maintained in this laboratory. Also supplementary choline produces a stimulation of the growth rate in these animals while it fails to do so in our strain.[5] The experimental period was 21 days. All rats received orally 0.1 cc of U.S.P. XI cod liver oil and 0.1 cc of a solution containing 25 γ of thiamine, 20 γ of riboflavin, 100 γ of calcium pantothenate, 100 γ of nicotinic acid and 20 γ of pyridoxine per day. The diets and distilled water were available *ad libitum*. The food intake was determined daily and the weight changes recorded 3 times weekly. The livers were removed from the animals under pentobarbital anesthesia and analyzed for total lipids.[6] In the table the weight changes are recorded as percentage change from the initial weight and liver fat is given in terms of g of fat per 100 g of moist liver, and per 100 g of body weight.

Compounds. With one exception the compounds tested were introduced into the basal diet at the expense of the glucose. The dimethyl sulfide was dissolved in corn oil and injected intraperitoneally each day in an amount of 7 mg per rat. The choline, inositol, betaine, and dimethyl sulfide were commercial products of acceptable purity. The S-methyl-

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2 Eckstein, H. C., and Singal, S. A., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 512.

3 Best, C. H., and Ridont, J. H., *Ann. Rev. Biochem.*, Stanford University, 1938, **7**, 349.

4 Van Prohaska, J., Dragstedt, L. R., and Harms, H. P., *Am. J. Physiol.*, 1936, **117**, 166.

5 Treadwell, C. R., *J. Biol. Chem.*, 1945, **160**, 601.

6 Tucker, H. F., and Eckstein, H. C., *J. Biol. Chem.*, 1937, **121**, 479.

TABLE I.
Lipotropic Activity of Various Compounds Under Standardized Conditions.

Dietary supplement	%	No. of rats	Change in wt,† %	Food intake per day,† g	Liver lipids per 100 g: Moist liver,† g	Liver lipids per 100 g: Body wt,† g
Basal		10	17.6 ± 2.8	9.1 ± .5	19.1 ± 2.5	1.02 ± .20
Lipociac	0.1	8	17.3 ± 1.7	10.5 ± .4	20.5 ± 2.1	1.06 ± .18
S-ethylcysteine	0.1	7	24.3 ± 2.5	10.2 ± .3	18.6 ± 1.4	.97 ± .11
Cystine betaine	0.1	4	26.4 ± 5.4	11.0 ± .2	17.4 ± 2.8	.92 ± .23
S-methylcysteine	0.1	8	19.8 ± 1.9	10.5 ± .3	17.3 ± 1.0	.91 ± .12
Inositol	0.1	7	26.4 ± 2.9	9.4 ± .2	16.0 ± 1.8	.73 ± .14
Dimethyl sulfide*		8	24.2 ± 3.6	9.7 ± .4	14.8 ± 2.1	.71 ± .20
S-methyl-isothiourea	0.2	7	0.9 ± 3.4	7.0 ± .3	11.5 ± 0.5	.62 ± .04
Betaine	0.1	7	27.9 ± 1.8	8.8 ± .4	12.8 ± 1.1	.52 ± .06
Choline	0.05	8	30.9 ± 5.7	10.3 ± .6	12.3 ± 1.2	.49 ± .07
Choline	0.1	7	30.2 ± 2.5	9.8 ± .5	11.0 ± 1.8	.41 ± .04
Triethyl-choline	0.1	8	23.4 ± 3.6	10.7 ± .4	10.2 ± 1.2	.41 ± .06
Lipociac	1.0	7	39.1 ± 1.9	12.2 ± .3	8.5 ± 0.4	.30 ± .01

* See text

† Including the standard error of the mean calculated as follows: $\sqrt{\Sigma D^2/N - 1}/\sqrt{N}$

isothiourea sulfate,[7] cystine betaine,[8] S-methylcysteine,[9] S-ethylcysteine,[9] and triethylcholine[10] were synthesized in this laboratory and shown by analysis to be of satisfactory purity. The lipociac was prepared from fresh pork pancreas according to the directions of Clark, Eilert and Dragstedt.[11] It contained 9.77% nitrogen, 1.03% methionine, and .92% cystine. The material gave a negative test for choline with ammonium reineckate both before and after hydrolysis.

Results. The results are shown in Table I. They are arranged in order of decreasing liver fat per 100 g body weight. The choline-containing diets were included for comparative purposes. The lipociac (0.1%) and S-ethylcysteine did not influence the level of the liver fat while the S-methylcysteine and the cystine betaine gave small decreases of questionable significance. Inositol, dimethyl sulfide, and S-methylisothiourea sulfate were intermediate in effect. Choline, betaine, triethylcholine, and lipociac (1%) produced the greatest lowering of the liver fat. The amounts of the substances to be incorporated in the diets were chosen to give a submaximum effect so as to eliminate possible side effects of quantities in excess of that utilized in lipotropism. With none of the experimental diets was the liver fat within the normal range (0.15-0.30 g per 100 g body weight) except that containing 1% lipociac. The results with lipociac at the 1% dietary level are especially interesting. Its lipotropic activity was as great or greater than 0.1% choline and in addition it gave the greatest stimulation of growth. The 1% lipociac supplied 1.26 mg of methionine and 1.12 mg of cystine per rat per day. It has been shown that methionine is approximately 1/5 as active as choline in decreasing the liver fat,[1] and the animals receiving choline (0.1%) in this experiment were ingesting 9.8 mg per day which produced a liver fat of 0.41 g per 100 g body weight. Thus it seems quite unlikely that the methionine content of the lipociac was sufficient to account for the lipotropic effect or the stimulation of growth. Moreover, in order to explain the lipotropic activity of the lipociac on the basis of choline as a contaminant, the lipociac would have had to contain approximately 10% choline which certainly would have been detected by the reineckate procedure. These effects of lipociac are being further investigated. The marked depression of the growth rate by S-methyliso-

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[8] Schubert, M. P., *J. Biol. Chem.*, 1935, **111**, 671.

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[11] Clark, D. E., Eilert, M. L., and Dragstedt, L. R., *Am. J. Physiol.*, 1945, **144**, 620.

thiourea sulfate is also of interest and suggests that further investigation with this compound might be profitable. It is possible that the effect of S-methylisothiourea sulfate on growth is due to the phenomenon of metabolic antagonism.

Summary. A representative group of substances, previously reported to be lipotropically active, have been tested under standardized conditions. Under the present experimental conditions and in the amounts fed, S-ethylcysteine did not decrease the level of the liver lipids, cystine betaine and S-methylcysteine exhibited a slight activity of questionable significance, inositol, dimethyl sulfide and S-methylisothiourea sulfate were of intermediate activity, and betaine and triethylcholine had approximately the activity of choline. Lipociac, at the 1% dietary level, was highly active lipotropically and stimulated growth to a greater degree than any of the other compounds tested. S-methylisothiourea sulfate depressed the growth rate.

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Effects of Temperature and Ultraviolet Light on Experimental Polyarthritis of Rats.*

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The experimental polyarthritis produced by the L-4 strain of pleuropneumonia-like microbes in rats is not strictly comparable to rheumatoid arthritis in man. However, because it responds to gold therapy similarly to rheumatoid arthritis, we consider it a method for studies of factors which might influence the arthritic processes and for making chemotherapeutic trials in animals.

In this study attempts were made to determine whether changes in environmental temperature or in ultraviolet irradiation would alter the course of the disease. This is of importance since, in man, it has commonly been supposed that changes in temperature and ultraviolet irradiation would alter the course of rheumatoid arthritis and the incidence of rheumatic fever. Therefore, we decided to evaluate the effects of these environmental changes with the object of increasing basic knowledge regarding the natural history of the disease in the rat.

Exposure. Thirty male and 30 female albino rats weighing 100 g were placed in an outdoor shelter with open screen sides which protected them from wind, rain and sunshine but not from changes in temperature. The nocturnal temperature ranged from 30 to 45°F, mean 37°F; the diurnal temperature ranged from 46 to 84°F, mean 54°F, the overall range being 30 to 84°F, mean 45°F. The temperature records were made on an automatic thermograph. The average relative humidity was 86% at 5 A.M., 64% at noon, and 70% at 5 P.M.‡ The diet used was Purina Dog Checkers (compressed pellets containing protein 21%; fat 4%; fibre 6%; nitrogen-free extract 46%; and ash 9%). All animals were allowed free access to water. For one week prior to inoculation with pleuropneumonia-like organisms (P.L.O.§) the animals were subjected to exposure. They were then given intraperitoneally 2 cc of a

* This work was supported, in part, by contract with the Office of Naval Research, U. S. Navy Department, and, in part, by the Stern Fund for Research in Arthritis.

† With the technical assistance of Selig A. Gellert and Pelagio S. Tabar.

‡ Official figures supplied by U. S. Weather Bureau, San Francisco.

§ Abbreviation used throughout to designate L_4 strain of pleuropneumonia-like organisms.

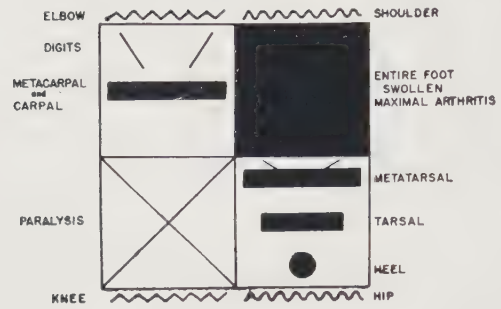

FIG. 1.
Athrogram for recording the extent of joint involvement in experimental polyarthritis of rats.

24-hour broth culture of P.L.O. The animals were examined for gross evidence of arthritis at the end of 4 days, daily for the next week, and then weekly for the following 6 months. The extent of arthritis was recorded on an arthrogram (Fig. 1) which was a modication of Sabin's arthogram.[1] For scoring purposes numerical values were assigned corresponding to the extent of arthritic involvement. The score values ranged from 0 to 4 for each anterior extremity and from 0 to 5 for each posterior extremity; thus the arthrogram score for any individual rat lay within a range of 0 to 18, 0 indicating no demonstrable arthritis and 18 indicating all 4 extremities were involved to a maximum degree. Single composite arthrogram scores were calculated for each animal and group of animals to represent the maximal arthritic involvement during the 6-month period of observation. Ten animals of each sex were uninoculated and kept as controls. In addition to the 20 controls, 20 males and 20 females were inoculated and kept in the animal room as controls where the average daytime temperature was 70°F and the night temperature 58°F.

The rats exposed to cold responded by developing a heavy growth of fur; however, 3 of the uninoculated controls died presumably from the effects of exposure. In the inoculated group, 35% of the females and 40% of the males died, while only 80% and 65%, respectively, showed arthritis. There appeared to be no loss of body weight attributable to the change in environment. The severity of arthritis according to the composite arthrogram score was slightly less than that of the inoculated animals kept at 70°F and the uninoculated controls subjected to cold did not develop arthritis, but only 85% survived.

Heat. Eight female albino rats weighing 100 g were placed in a heated cabinet with an average temperature of 102°F for 24 hours with little variation. The animals were inoculated as described above with P.L.O. They had the same diet but were given thiamine hydrochloride in the drinking water, since rats kept at high temperatures rapidly develop thiamine deficiency. The animals kept at 70°F served as controls for this group.

Immediately after the animals were placed in the heated cupboard they ate less for 3 or 4 days and thereafter resumed normal food consumption. After 6 months of heating these animals had gained normally in body weight, and their tails became longer than those of the controls. Ten heated males died before the scheduled inoculation. Eight heated females, which were inoculated, showed composite arthrogram scores slightly less than for the inoculated controls kept at 70°F. Only 88% of these females developed arthritis as compared with 95% for the controls. The total mortality of these females was only 12% as compared with 15% for the inoculated controls, 35% for those subjected to exposure, and 50% for the females subjected to ultraviolet light.

Ultraviolet Irradiation. Thirty male and 30 female albino rats were exposed for 6 months to constant ultraviolet irradiation supplied by a Westinghouse Type S-1 ultraviolet lamp at a distance of 5 feet. The average temperature remained constant at 82°F. Ten animals of each sex were used as uninoculated controls.

A few of the irradiated rats developed a mild conjunctivitis, but there was no increase in body weight or food consumption. Among the uninoculated controls subjected to ultraviolet light one animal died of undetermined cause and none developed arthritis. Among the inoculated animals the females had higher and the males lower composite arthrogram

[1] Sabin, A. B., and Warren, J., *J. Bact.*, 1940, **40**, 823.

TABLE I.
Influence of Temperature and Ultraviolet on Experimental Polyarthritis of Rats.

	Controls* (70°F)		Exposure† (30 to 84°F)		Ultraviolet‡		Heat§ (102°F)
	Female	Male	Female	Male	Female	Male	Female
Composite arthrogram score	2.9	4.2	2.5	2.3	3.3	1.9	2.6
% showing gross joint involvement	95	100	80	65	75	60	88
% dead within 4 days	5	0	15	10	10	5	0
% total deaths	15	10	35	40	50	45	12
% dead or infected	100	100	95	70	100	90	88
% showing no symptoms	0	0	5	30	0	10	12

* Total No. of rats, 40.

† Exposure controls: 3 deaths were probably due to exposure, leaving 85% survived; total No. of uninoculated rats, 20; No. of inoculated rats exposed to cold, 60.

‡ Ultraviolet controls: No arthritis developed. One animal (male) died of undetermined cause; total uninoculated rats, 20; total inoculated and irradiated rats, 60.

§ Total, 8 rats.

scores than the inoculated controls kept at 70°F and not subjected to additional ultraviolet light. Only 75% of the females and 60% of males developed arthritis as compared with 95% and 100%, respectively, among the inoculated controls. The death rate was higher than that of the inoculated controls, being 50% for females and 45% for males as compared with 15% and 10%, respectively.

Conclusion. In the experimental polyarthritis of rats produced by the L_4 strain of pleuropneumonia-like organisms exposure to cold or increased ultraviolet light under the conditions used caused increased mortality but resulted in a smaller incidence and severity (females only) of arthritis; whereas, increased heat caused little deviation in mortality and arthritis involvement from the inoculated controls.

16820

Protein Intake and Leishmaniasis in the Hamster.*

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It has already been shown that the course of a parasitic disease may be altered by the level of protein intake of the host,[1-2] low levels of intake decreasing host resistance.

Leishmaniasis in the hamster was chosen for the present study because *Leishmania donovani,* the causative agent of kala-azar, a highly fatal human disease is an intracellular parasite living and reproducing in the vertebrate host within the cells of the so-called reticulo-endothelial or lymphoid-macrophage system.

Blockade of the R-E system,[3] *e.g.* with india ink, has been shown to result in a lowering of the resistance of the animal involved; but, to be effective, repeated injections of ink are necessary. The growth and reproduction of leishmania in the R-E cells is believed to constitute a functional blockade of this

* This work was supported in part by the Protein Metabolism Fund of the Bureau of Biological Research.

1 Seeler, A. O., and Ott, W. H., *J. Inf. Dis.,* 1945, **77**, 181.

2 Seeler, A. O., and Ott, W. H., *J. Nat. Malaria Soc.,* 1946, **5**, 123.

3 Jaffe, R. H., *Physiol. Rev.,* 1931, **11**, 277.

system with increasing numbers of parasites matching the compensatory hyperplasia of the R-E cells.

The problem under study was to learn the effect of such an endogenous blockade of the R-E cells on animals fed diets deficient in protein as well as on animals fed diets containing excess of protein.

Materials and methods. The Syrian hamster, *Cricetus auratus,* was chosen as the host because of its proven susceptibility to *L. donovani* and because the course of leishmaniasis in it is similar to that seen in man. Infection was obtained by the intraperitoneal inoculation of amounts of a saline suspension of hamster spleen, ground in a Ten Brock tissue grinder, suitable to produce severe infection in 60-100 days. The usual dose was equivalent to 20 mg of a heavily infected spleen. Aseptic precautions were observed in the transfer of the infection. The parasite used was of the Khartoum strain and obtained by us from Dr. A. P. Richardson of the Squibb Institute for Medical Research.

TABLE I.
Composition of the Hamster Diets Used.

Basic Protein (20%)	g
Casein	200
Primex or Crisco	250
Corn Oil	20
Cerelose	200
White Dextrin	257
Salt Mixture*	40
Cellu Flour	20
A, D, and E conc.†	1
Choline Chloride	2
Wilson's 1:20 liver powder‡	10
	1000

To each 1000 g add 0.372 g of vit. B mixture§ and 0.010 g vit. K.

Low Protein (10%)
Substitute in above:
100 g Casein
357 g Dextrin

High Protein (40%)
Substitute in above:
400 g Casein
57 g Dextrin

* Wesson, *Science,* 1932, **75**, 339.

† A, D, and E concentrate:

	g
Corn oil	41
A and D conc. (450,000 U. S. P. units of A) (90,000 U. S. P. units of D)	7
Alpha tocopherol	2
	50

‡ Armour's liver extract (23 ml) used in place of liver powder.

§ Vit. B mixture:

	mg
Thiamine hydrochloride	200
Riboflavin	400
Pyridoxine HCl	200
Niacin	1000
Calcium pantothenate	1100
Para-aminobenzoic acid	1000
Inositol	5400
	9300

The principal diet used was a modified mouse diet† (Table I). Although niacin is apparently not necessary in the diet of the hamster[4] it was included here since animals on protein deficient diets may not have the same requirements as animals on control diets. One or two weeks prior to inoculation all animals were placed on their test diets containing either high (40%), basic (20%) or low (10%) levels of protein. The protein used was casein. The high and basic protein diets allowed immature hamsters to gain weight at approximately a normal rate. All groups of hamsters ate the diet well.

Results. The results obtained may be summarized under the headings of survival, body weight changes, estimated number of parasites and organ weight changes.

Infected animals survived longer when maintained on the high and basic protein diets than on the low protein diet. The animals on the low protein diet (Fig. 1) were all dying when the experiment was terminated while those of the other 2 groups were in fairly good condition. Fig. 1 shows also that the fall in weight of the animals on the low protein diet to a value below their initial weight is a terminal phenomenon. High intake of protein, therefore, partially protects against the progressive emaciation characteristic of the disease.

Although this "protective" effect of the basic and high protein diets seems to be borne out by the relative numbers of parasites in the impression smears made from the

† Details of diet furnished in personal communication through courtesy of Dr. David Bosshardt, Sharp & Dohme Co.

4 Cooperman, J. M., Waisman, H. A., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 250.

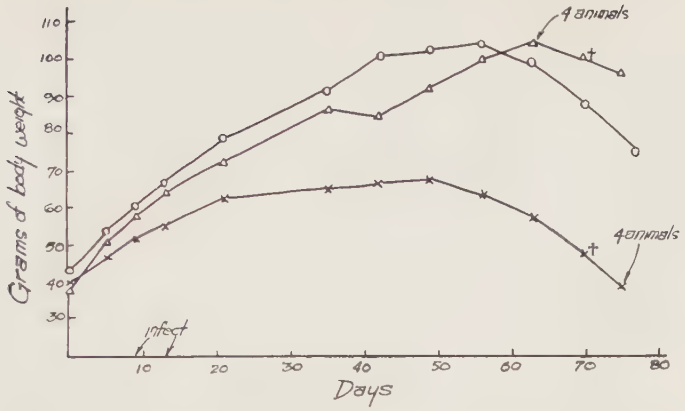

FIG. 1.
Weight curves of immature hamsters, infected with *Leishmania donovani*, fed experimental diets of different protein content: Δ High protein diet (40% casein); ○ Basic protein diet (20%); × Low protein diet (10%).

TABLE II.
Effect of Low, Basic and High Protein Diets on Organs and Organ-weight, Body-weight Ratios; and on the Parasite Counts of Growing Hamsters Infected with *Leishmania donovani*. All figures represent average values for the group. All animals were sacrificed between 75-77 days after being placed on the diets.

Conc. of protein in diet	No. of animals	Body wt (g)	Spleen wt (mg)	Spleen-wt body-wt ratio*	Liver wt (mg)	Liver-wt body-wt ratio*	Estimated No. of parasites
Low (10%)	5	38.1†	209.2†	5.3†	3248†	85.4†	++++
Basic (20%)	5	75.2	488.6	6.5	5143	68.3	++
High (40%)	5	96.2†	840.8†	8.7†	7097†	73.8†	+±

* Ratios expressed as milligrams of organ per gram of body weight.
† Averages represent only 4 animals of the group. One animal died before 75th day.

spleen at necropsy (Table II), when calculated on the basis of the estimated absolute numbers of parasites in the enlarged spleens the differences are much smaller. Indeed, the data seem to suggest that the reproductive rate of the leishmania may be nearly constant on all 3 diets.

The pathology observed was essentially that described by Meleney[5] all heavily-infected animals showing marked splenomegaly and hepatomegaly, both absolute and relative to body weight (Table II and Fig. 2). The liver-weight/body-weight ratio of the normal hamster is approximately 43 mg/g (range of ratio from 40-46 for animals 85-124 g body weight). The ratio in the infected animal usually does not increase quite 100% in value. The normal spleen-weight/body-weight ratio is about 1.4 mg/g for animals weighing from 85-124 g. In the infected

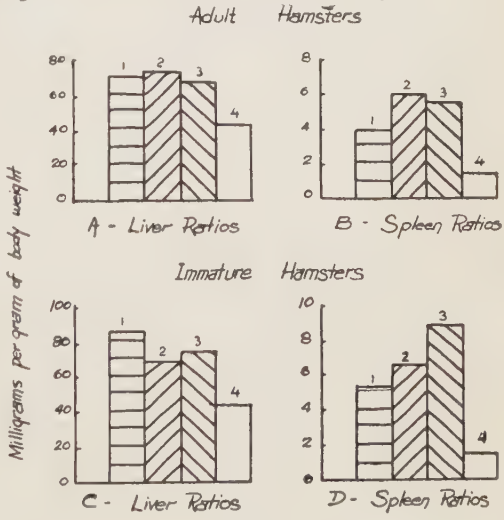

FIG. 2.
Organ weight, body weight ratios for adult and immature, infected and uninfected hamsters fed diets differing in protein content. 1, Low protein diet, infected; 2, Basic protein diet, infected; 3, High protein diet, infected; 4, Stock diet, uninfected.

[5] Meleney, H. E., *Am. J. Path.*, 1925, **1**, 147.

hamster this usually increases more than 300%. The spleen, therefore, is a better index of infection. Except possibly, for the relatively low ratios for the spleens of animals on low protein diets there are probably no significant differences in organ weight ratios under the dietary conditions studied.

Work is now in progress to determine the effect of kala-azar on the liver nitrogen in growing hamsters.

Summary. Protein intake influences the course of leishmaniasis in the hamster, deficient diets leading to earlier emaciation and death. Excess dietary protein seems to favor survival.

16821

Serum Cholinesterase in Some Pathological Conditions.*

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Of the esterases in human blood, the one hydrolyzing acetylcholine into acetic acid and choline has received the most attention in recent years because of the supposed relationship of this enzyme to the physiology of nerve activity. Actually, recent work indicates that there are two enzymes involved. One, the so-called true or specific cholinesterase, is thought to be involved directly in the transmission of nerve impulses, whereas the non-specific or pseudocholinesterase has, as yet, no assigned function.

Since the activity of the latter enzyme is easily measured, and since it is present in a readily available material (serum) in hospital patients, it offers an opportunity to study variations in a basic enzyme system in a number of pathological conditions. Such variations in themselves may be of more importance when the nature and function of the enzyme itself is better understood, nevertheless these variations are a guide to alterations in the metabolism of the organ or organs producing, distributing and disposing of the enzyme.

We have confirmed the findings of those workers who have noted a great spread in the values for pseudocholinesterase in the serum of normal individuals. This may be seen in the summary of normal values in the tables which follow. This spread has confused many observers who have been unable to come to any conclusion as to variations from the normal of serum cholinesterase in numerous pathological conditions. However, such difficulties are obviated by using sufficiently large samples thus permitting statistical analysis.

Of the published information available, there is evidence to indicate a decrease in the value of the enzyme in liver damage[1–6] and in pernicious anemia in relapse.[7,8] We have observed the decrease in cases of liver damage

* Aided by a grant from the Cancer Research Grants Division, U. S. Public Health Service.

† With the technical assistance of Anita A. Suran, who was assisted in part by a fellowship from the California Tuberculosis and Health Association, Medical Research Committee.

‡ We are grateful to Dr. Emil Bogen, Olive View Sanatorium, for assistance in obtaining sera for our tuberculosis studies.

1 Antopol, W., Tuchman, T., and Schiffren, A., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 46.

2 Antopol, W., Schiffren, A., and Tuchman, L., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 363.

3 McArdle, B., *Quart. J. Med.*, 1940, **9**, 107.

4 Faber, M., *Acta Med. Scand.*, 1943, **114**, 72.

5 Kunkel, H. G., and Ward, S. M., *J. Exp. Med.*, 1947, **86**, 325.

6 Wescoe, W. C., Hunt, C. C., Riker, W. F., and Litt, I. C., *Am. J. Physiol.*, 1947, **149**, 549.

7 Sabine, J. C., *J. Clin. Invest.*, 1940, **19**, 833.

8 Meyer, L. M., Sawitsky, A., Ritz, N. D., and Fitch, H. M., *J. Lab. and Clin. Med.*, 1948, **33**, 189.

due to a variety of causes. Since this phenomenon has been reported comprehensively in the literature, we are not at this time publishing our data. In three other disease states, either covered not at all or inadequately by previous workers, we have made observations which we are reporting here.

Serum cholinesterase in pregnancy. Butt and co-workers[9] found in 14 women (who were either pregnant at the time or had recently given birth) that the "readings obtained in the course of pregnancy fall within the limits of variation of normal, but with two exceptions they are all below the mean found for normal females." Hall and Lucas[10] and Milhorat[11] in small series found no variation from the normal in pregnancy. Laborit and Morand[12] observed a decrease in serum cholinesterase during labour but an increase during pregnancy and Davis *et al.*[13] found the enzyme to remain normal during pregnancy. Finally, Zeller and associates[14] found no statistical deviation from normal of the enzyme in pregnancy.

In light of the above contradictory findings, we first ran a series of pregnant women at the time of delivery. The following reagents were employed in terms of the final concentration in a total volume of 3 cc:

.1 cc serum
.0017 M. sodium bicarbonate
.0034 M. acetylcholine chloride

The production of acetic acid from acetylcholine was followed at 37° by means of the liberation of carbon dioxide from a bicarbonate-carbonic acid buffer in Warburg vessels. These vessels were gassed at room temperature with a mixture of 95% nitrogen and 5% carbon dioxide. At zero time, the acetylcholine was tipped into the serum and bicarbonate mixture. Readings were taken at 20 and 40 minutes. Since the two are comparable only the latter is given in Table I.

We may calculate from Table I that the difference between the means is 15 μl. CO_2 and the probable error of the difference is 1.81 μl of CO_2 This difference is 8.28 times the probable error (a significant difference, occurring by chance only once in over 15 million trials).

In order to verify the above observations, we repeated the experiment under new conditions. This time the reagents were identical to those used by Mazur and Bodansky[15] who followed essentially the method described by Ammon.[16] In the final reaction mixture of 3 cc, the concentration of the acetylcholine was 0.015 M (pH 7.7). The enzyme was present in .5 cc serum diluted 1:10 in the $NaHCO_3$. The remainder of the procedure is the same as described above. The results for 20 minutes are listed in Table II.

The statistical significance of the data in Table II is evident from the figures on the magnitude of the differences between the means of the pregnant groups and the normal group and the probable error of these differences. If the difference between the means is 10 times the probable error of this difference, the probability of this occurring by chance is 65 billion to one.[17] It is obvious, therefore, that there is a decrease in the serum cholinesterase during pregnancy, and also at the time of delivery.

Serum cholinesterase in tuberculosis. There are a few references to serum cholinesterase in tuberculosis in the medical literature.[18,19] None, however, has a sufficiently large series

9 Butt, H. R., Comfort, M. W., Dry, T. V., and Osterberg, A. E., *J. Lab. and Clin. Med.*, 1942, **27**, 649.

10 Hall, G. E., and Lucas, C. C., *J. Pharm. and Exp. Ther.*, 1937, **59**, 34.

11 Milhorat, A. T., *J. Clin. Invest.*, 1938, **17**, 649.

12 Laborit, H., and Morand, P., *Gynecologie et Obstetrique*, 1947, **46**, 298.

13 Davis, M. E., Si-Feng Yu, E., and Fugo, N. W., *J. Clin. Endocrinology*, 1948, **8**, 666.

14 Zeller, E. A., Birkhauser, H., Wattenwyl, H. V., and Wenner, R., *Helv. Chim. Acta*, 1941, **24**, 962.

15 Mazur, A., and Bodansky, O., *J. Biol. Chem.*, 1946, **163**, 261.

16 Ammon, R., *Arch. ges. Physiol.*, 1930, **233**, 486.

17 Pearl, R., Medical Biometry and Statistics, Saunders, Phila., 1940.

18 de Michele, G., *Boll. Soc. Ital. Biol. Sper.*, 1944, **19**, 66.

19 Crestol, P., Passovant, C., Benezech, C., and Dutarte, G., *Presse Med.*, 1946, **54**, 557.

TABLE I.
Serum Cholinesterase* in Normal Women and Pregnant Women at the Time of Delivery.

	No. of individuals	Age range	Mean value* in μl CO_2	Stand. Dev.
Normal	67	18-40	90	±17
Pregnant	59	18-40	75	±13

* 40 minutes at 37°C and calculated to 760 mm mercury at 0°C.

TABLE II.
Serum Cholinesterase in Pregnant and Normal Women.

Group	No. of individuals	Age range	Mean value in μl CO_2*	Stand. Dev.	Δ
Prenatal 1-6 months	48	18-40	65.9	±13.9	10.4
" 6-9 "	38	18-40	64.6	±13.0	10.8
At term	52	18-40	64.3	±19.6	9.5
Normal women	80	18-40	86.7	±19.5	

* 20 minutes at 37°C and calculated to 760 mm mercury at 0°C.

$$\Delta = \frac{\text{Difference between the means}}{\text{Probable error of this difference}} = \frac{}{\sqrt{(\text{P.E.}_{M1})^2 + (\text{P.E.}_{M2})^2}}$$

TABLE III.
Serum Cholinesterase in Normal People and Individuals with Pulmonary Tuberculosis.

Group	No. of individuals	Mean	± Stand Dev.	Δ
T.B. men	60	82.7	26.5	4.1
Normal men	66	94.2	19.2	
T.B. women	83	80.9	28.5	2.2
Normal women	80	86.7	19.5	

to warrant statistical analysis. We were fortunate in being able to obtain a fairly large number of sera from patients with pulmonary tuberculosis at the Olive View Sanatorium, Olive View, California. A total of 60 men and 83 women were studied. These had been previously classified clinically as follows:

	Total	Advanced	Moderate	Mild
Men	60	50	9	1
Women	83	62	16	5

Serum cholinesterase was determined by the method described above for the second series of pregnant women. The results for tuberculosis are listed in Table III.

From Table III, it may be seen that since Δ is only 4.1 in the comparison of tuberculous and normal men, the probability of this occurring by chance is only 174 to one. In the case of the women, Δ being only 2.2, the probability of this occurring by chance is 6.25 to 1. We do not feel that these figures are significant. Hence, in our series, we may conclude that pulmonary tuberculosis does not alter the serum cholinesterase values.

Serum cholinesterase in patients with neoplasms. Greenstein[20] has reviewed in a comprehensive fashion variations from normal of enzyme values in tumor bearing hosts. Although the discovery of such variations has not proved to be of diagnostic value, we agree with Greenstein as to their basic importance: "More knowledge is needed on the analytical chemistry of the cancer cell, particularly of its enzymes and coenzymes, of the non-catalytic proteins, of the sugars, nucleic acids, fats and salts—of their analytical distribution, rate of metabolic exchange and source of origin. To an equal extent, the same can be said of normal tissues; the intensive study of these tissues is bound to be helpful in research on tumors if only by stimulation, by the illumination offered by contrasting properties, and by providing new tools and approaches."

There is no previous statistical evidence of

[20] Greenstein, J. P., Biochemistry of Cancer, Academic Press, New York City, 1947.

TABLE IV.
Serum Cholinesterase in Patients with Malignant Tumors.

Site and type primary tumor	Metastasis	μl CO_2-20 min.
Squamous cell carcinoma hand	+	33.1
" " " tongue	—	63.6
" " " face	—	90.0
" " " larynx	—	58.1
Adeno-carcinoma rectum	—	63.8
" " "	+	47.5
" " stomach	—	53.8
" " "	—	50.1
" " "	—	39.2
" " "	+	56.5
" " "	+	39.2
" " "	+	53.7
" " prostate	—	33.9
" " uterus	—	52.7
Transitional cell carcinoma bladder	—	73.6
" " " "	—	68.3
" " " "	—	78.3
" " " "	—	83.7
Myeloma femur	+	54.2
Reticulum cell sarcoma	—	59.3
	Mean =	62.7
	Standard deviation =	±18.8
	Δ =	8.3

variation in serum cholinesterase in patients with neoplasms. We have studied 26 cases of men and women with a variety of malignant tumors. The reagents and methods were the same as described for pregnant women in Table II.

The Δ value of 8.3 given in Table IV is obtained by comparing the mean for the cancer group with the mean for normal women. This gives a value lower than the true one since the cancer group contains both men and women. However, even with this lower value the probability of the difference between the means occurring by chance is over 15 million to one. We must thus conclude that there is a drop in serum cholinesterase in patients with malignant tumors.

Summary. There is a significant decrease in serum cholinesterase in women in the various stages of pregnancy and in patients with neoplastic disease. There is no decrease in our series in patients with pulmonary tuberculosis.

16822

Enumeration of Virus Particles by Electron Micrography.*

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In the study of viruses or other particulate materials in aqueous suspension with the electron microscope, it is necessary to secure a thinly spread deposit of the particles on the collodion film. This collodion film is then studied by transmitted electrons either in light or dark field illumination, or the particles on the film may be metal shadowed by the method of Williams and Wyckoff[1] before study. In any case it is desirable that each kind of particle in the suspension be present on each area of the collodion forming one complete field in the electron microscope. It is desirable also that the aggregates, if any, of the particles seen in the microscope should not be produced in the procedure by which the particles were deposited on the film; that is, any aggregation observed should be representative of the state of dispersion of the particles present in the suspension under study. Furthermore, it is highly desirable that the number of particles per unit area of the collodion (per microscope field) be the same from place to place and equal to the number in a known volume of the suspension under study.

At present there is no technic fulfilling these conditions. The usual procedure consists of placing a drop of the virus suspension on the collodion film and leaving it for a few seconds. The fluid is then withdrawn with a fine pipette, and the residual, thin film is left to dry and to deposit its particles on the collodion. Although it is sometimes possible to obtain suspensions of animal viruses in water, this is not the general rule. Most of these materials do not disperse well in water, and other laboratory studies on such viruses are carried out in some suitable buffered salt solution. It is frequently desirable that electron micrographs be obtained with such salt suspensions for study of the state of the virus under these conditions for comparison with other data, those from the ultracentrifuge, for example. However, when the residue of salt solution containing virus dries on the collodion film, changes occur in salt concentration and, probably, in pH as well. The liquid film does not dry evenly, and as a result of a combination of these factors, it is clear that any particular distribution of deposited virus bodies or aggregates among them is likely to bear little resemblance to the state of dispersion of the particles in the suspension from which they came. In this paper† a method has been used in the study of formolized swine influenza virus which promises to correct some of the ills described above.

Materials and methods. The method is based on the use of a specially designed cell fitting into the rotor of the air-driven analytical ultracentrifuge. The cell, shown in the drawing of Fig. 1, is similar to the one used for sedimentation velocity studies but with some modifications for the present purpose. As seen in Fig. 1, the cell itself consists of 2 lucite windows held apart by a lucite block in which there is a space of about 1 ml volume with trapezoidal sides coinciding with radii of the rotor. In preparation for filling the cell, one lucite window is put in place with a thin rubber gasket to prevent leakage and locked in with threaded aluminum ring and spanner wrench. A piece of cover slip glass coated with collodion is inserted at the bottom of the cell and the cell filled with the virus sus-

* This work was aided by a grant to Duke University from Lederle Laboratories, Inc., Pearl River, N. Y., and by the Dorothy Beard Research Fund.

1 Williams, R. C., and Wyckoff, R. W. G., Proc. Soc. Exp. Biol. and Med., 1945, **58**, 265.

† This work was reported at the meeting of the Electron Microscope Society of America held in Toronto, September 10, 1948.

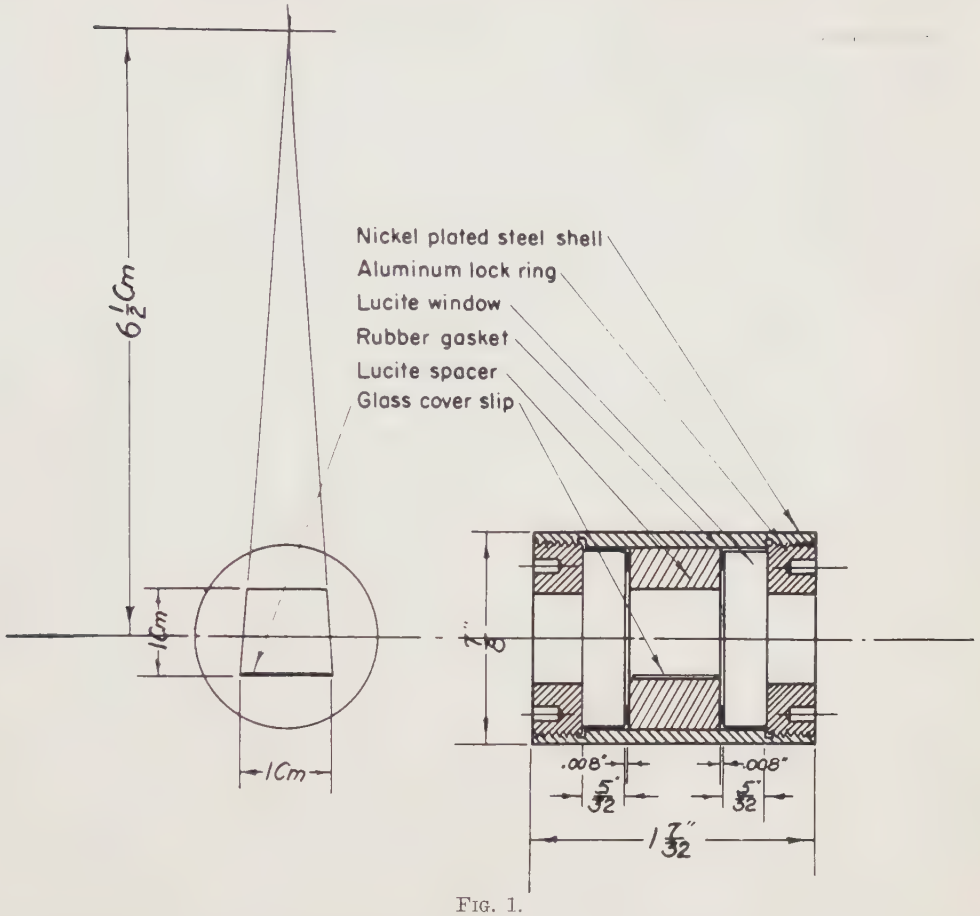

FIG. 1.

Construction details of the rotor cell for the analytical ultracentrifuge. In this cell the virus from about one ml fluid is sedimented onto the collodion-coated glass at the bottom. This glass is then removed; the collodion is stripped off; and the electron microscope is used to count and study the virus deposited upon it.

pension, after which the second window is placed and fastened with the corresponding aluminum ring. The cell is then inserted in the rotor of the analytical ultracentrifuge and, in the present experiment, spun at 16,500 g for 30 minutes; under such conditions the influenza virus particles should sediment, convection free, upon the collodion coating on the glass cover slip. After the run, the cell is opened and the cover slip carefully removed, dipped twice in distilled water to remove the salt and then dried. The film is scratched across with a needle and floated off the glass on a distilled water surface, The film, divided by the scratch, comes off in two pieces, on one of which is placed a standard 200 mesh electron microscope screen. The other piece is caught from below atop another such screen, this being the one with the virus particles on top for use in shadow casting.

Swine influenza virus was obtained from the chorio-allantoic fluid of virus infected chick embryos 4 years ago and formolized for the preparation of vaccines. The procedures for concentration and partial purification of the virus by ultracentrifugation have been described elsewhere.[2] This material was in Ringer solution in a stoppered tube stored in the refrigerator at 2 to 8°C.

Experimental. In the preliminary experi-

[2] Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, Dorothy, Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1944, **48**, 361.

ments virus preparations for electron micrography were made in the usual manner. The suspension of formolized virus was diluted one to ten and prepared for the electron microscope by drying a small amount on the collodion film. Such preparations yielded approximately 25 to 100 virus particles per cm^2 in pictures taken at a magnification of 4,500 x. This was about the concentration of virus ordinarily employed for electron microscope study of the purified virus.

In order, now, to produce pictures of controlled deposition of virus, the procedure departed from the usual methods. A further dilution of the virus suspension 1-100 (total dilution 1-1000) was made with physiological saline solution and this was put into the rotor cell described above and spun for 30 minutes at 16,500 g. Such preparations yielded uniform distribution of virus particles, and pictures were taken with and without shadow casting. The procedure was repeated using 1-2,000, 1-4,000, 1-8,000, 1-16,000 and 1-32,000 dilutions of the same starting material. When the series was finished, repeat runs were made on freshly made 1-4,000, 1-8,000 and 1-16,000 dilutions to check the original data.

All of the electron micrographs were made at a magnification of 4,500 x, and a grid of fine lines one centimeter apart was projected with the original negatives so that the prints would have a counting standard of area independent of photographic enlargement. About a hundred pictures in all were made of various areas of the films produced. The counts per cm^2, as well as the total number of particles counted, and the statistical variation expressed as standard deviation (σ) are shown for the various dilutions and for the repeat runs in Table I. Strips of representative pictures of the chromium shadow-cast preparations are shown in Fig. 2.

Although this procedure is more difficult than simple drying of the material in the usual way, it yields counts agreeing well with the dilution of the starting suspension. Variation in the number of virus particles per unit area is small, and for this reason, one can get a good general impression of the preparation from examination of only a few fields. Furthermore, the counts obtained from repeat runs at a given dilution showed gratifying similarity.

Calculations. Some calculations have been made to determine the number of virus particles to be expected per unit area in these pictures. For this it was necessary to know the weight of virus per ml of the suspension used, the average unhydrated weight of the particles, and the magnification in the picture. The nitrogen content measured on purified swine influenza virus from this laboratory was 9%.[3] For the present work, a sample of the swine influenza vaccine was sedimented at 16,500 g for 30 minutes, and the nitrogen content of the sedimented material obtained by difference between Kjeldahl measurements on the whole material and on the supernate of this run. The resulting value multiplied by the factor 11.1 gave 1.85 mg virus per ml of the starting material. The 1/1,000 dilution thus contained 0.00185 mg virus per ml.

The average particle size, density and water content were taken from previously published data on the freshly purified virus.[4] In order to check the applicability of these data to the formolized virus used in the present experiments, 4 sedimentation velocity runs were made, 2 in physiological saline solution and 2 in a solution of bovine serum albumin in physiological saline (density 1.046). In the first pair of studies the sedimentation rates of the formolized virus corrected to water viscosity at 20°C were 671 and 677 x 10^{-13}, and in the second pair the values were 379 and 377 x 10^{-13}. When these values were plotted against the densities of the respective suspending media, 1.004 and 1.046, it was seen that the formolized virus had a size and density the same as the analogous properties reported for the freshly purified virus.[4] This is taken to mean that no great error will be made in using for particle radius, the value 58.5 mμ; for wet density, 1.100; and for water content by weight, 39.2%.

[3] Taylor, A. R., *J. Biol. Chem.*, 1944, **153**, 675.

[4] Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, Dorothy, Beard, J. W., *J. Biol. Chem.*, 1945, **159**, 29.

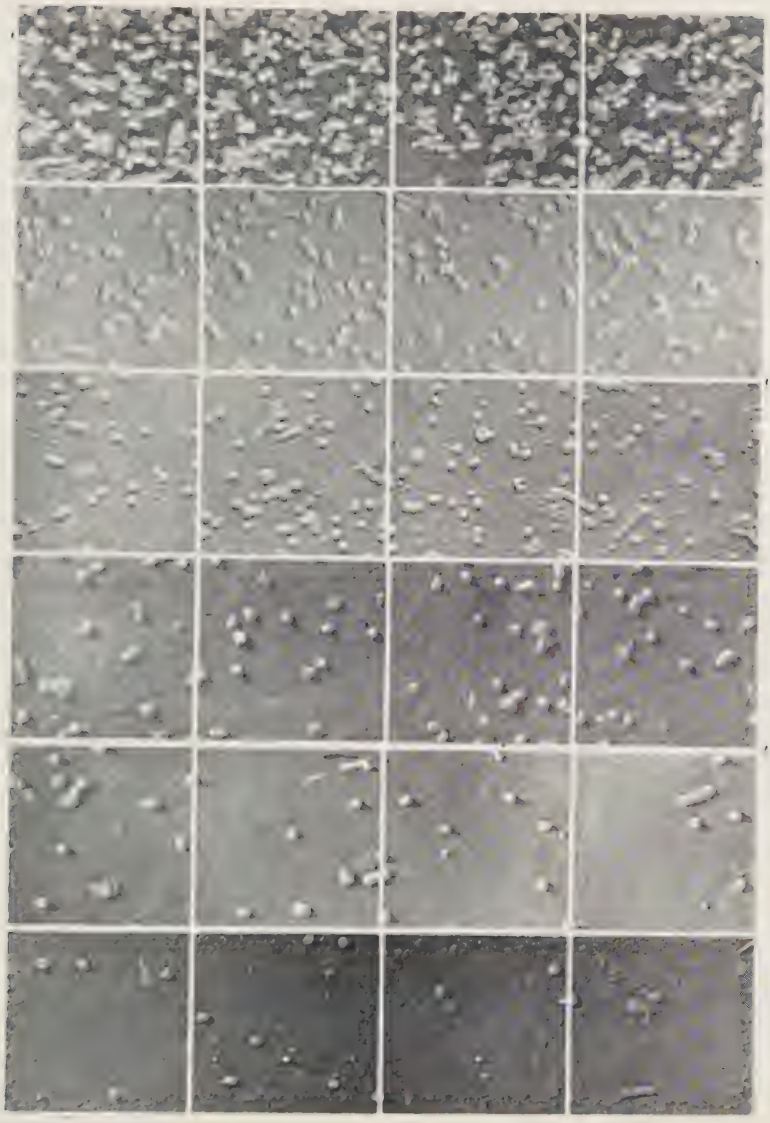

FIG. 2.

Influenza virus deposited upon a collodion membrane by sedimentation in the ultracentrifuge. These strips are representative of pictures taken of deposits from dilutions of 1-1,000, 1-2,000, 1-4,000, 1-8,000, 1-16,000, and 1-32,000 of the starting material. The data from the counts are given in Table I. The particles are shadow-cast with chromium at an angle of about 15°

At an electron magnification of 4,500 x, we can then calculate as follows:

$$\frac{4/3\ \pi\ r^3\ \rho\ (1—0.392)}{0.00000185 \text{ grams/ml}} = \text{g dry wt per particle.}$$

$$\frac{\text{g dry wt per particle.}}{4/3\ \pi\ r^3\ \rho\ (1 - 0.392)} = \text{particles per ml in the 1/1,000 dilution}$$

Equation 1.

where

r = hydrated virus particle radius (58.5×10^{-7} cm)

1.100 = ρ hydrated virus particle density.

1.85×10^{-6} = grams virus per ml in 1/1,000 dilution.

Because of the sector shape of the cell, the number of particles sedimented on unit area of its bottom is not simply the product of the area by the height but 7.1% less, an amount by which the trapezoidal sector area

TABLE I.

Dilution	Total particles counted		Particles observed per cm²		Particles expected Equation 2
	Run I	Run II	Run I	Run II	
1/ 1,000	5,420	—	121 ± 10*	—	151
1/ 2,000	3,418	—	61 ± 7	—	76
1/ 4,000	2,257	1,727	31 ± 6	34 ± 8	38
1/ 8,000	867	863	19 ± 5	16 ± 4	20
1/16,000	415	261	7 ± 2	7 ± 3	10
1/32,000	361	—	4 ± 2	—	5

* Variations are expressed as mean standard deviation. Standard deviation of the mean is of course much less and dependent on the number counted.

is less than that of a rectangle of the same height. The number of images per unit area of a micrograph at 4,500 x is then given by:

$$\frac{3 \times 1.85 \times 10^{-6} \times 0.929}{4\ \pi\ r^3\ 1.10\ (1 - 0.392)\ (4{,}500)^2} = 151$$

Equation 2.

The numbers expected for dilutions higher than 1/1,000 are correspondingly less. Clearly these calculated numbers are strongly dependent on accurate knowledge of particle size and electron magnification, for these enter the equation in third and second power respectively. The influence of the other variables is correspondingly less, and it can be judged from Equation 2.

Discussion. From the results shown in Fig. 2 and Table I, it is clear that this method makes possible the counting of the particles of swine influenza virus, yielding numbers closely proportional to the dilution factor of the starting material. Furthermore, agreement is quite good between these numbers and those predicted from calculations involving not only the physical characteristics of the virus particles but also the purity of the starting material. The observed numbers are lower by about 19% than the predicted ones as shown in Table I. Although error in several places may account for this, one source of error is immediately suspected. Not all of the sedimented virus particles might actually remain on the membrane through washing to appear in the final pictures.

The distribution of particles over the collodion membrane as seen in the electron microscope is exceedingly uniform and the pictures obtained in the process come from dilutions of virus 100 to 3,200 greater than those considered optimum for microscopy using standard technics. The advantages of such a method need not be discussed at length. If the results are proved by subsequent work to be strictly quantitative, the possible applications of the procedure are many, not the least of which should be to the study of viruses in preparations of such low virus content that too little of the agent would be present for purification and concentration, and in which the concentration is so low that the usual technics of preparation for microscopy would produce too few virus particles per field to be recognized.

For study of aggregation in suspension, this method should be useful not only for viruses but also for any other suspended material, such as bacteria in liquid media. For such large bodies as bacteria, any horizontal centrifuge carrying a suitable cell could be used for sedimentation of the particles directly on a microscope slide where, after removal from the centrifuge, they could be studied with a light microscope. It would be necessary to calculate the number of pairs, triplets, etc. to be expected from chance sedimentation of two or more particles on the same area of the slide; but when this number is subtracted, the excess would be characteristic of the liquid suspension. The author is not aware of any other satisfactory method of studying this problem.

Summary. A method has been devised for counting virus particles in a suspension by means of the electron microscope. Studies on formolized purified swine influenza virus showed that the particles could be sedimented with a uniform distribution on a collodion membrane in the ultracentrifuge as revealed by electron micrographs of the particles on the

membrane. The findings showed a close correlation with the dilution factor and with the number of particles calculated from chemical and physical data obtained with the virus.

16823

Electronmicroscopy of Cells from Tissue Cultures Infected with Vaccine Virus.

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One of the obvious needs in electronmicroscopy is a satisfactory method for good visualization of intact cells to provide information on their internal structure and the presence of virus particles in infected preparations. Progress in this field has been made in the use of specially prepared histological sections[1] and in the studies of individual avian cells cultured on plastic membranes.[2,3] The present report describes recent observations on the intracellular growth of vaccine virus observed by electronmicroscopy with a modification of the plastic membrane technic.

Materials and methods. The technic for cultivation of mammalian epithelial cells on plastic membranes and their preparation for visualization by the electron microscope has been described in detail elsewhere.[3-5] In brief, this is as follows: .02 cc of a 0.6% solution of "Formvar" (Shawinigan Falls Products Co., Shawinigan Falls, Quebec, Canada) in ethylene chloride is dropped into a petri dish full to overflowing with chilled Ringer's solution. A cover slip held by forceps is inserted under the membrane covering the fluid in the petri dish and the cover slip is raised through the membrane; this provides a smooth non-adherent plastic coating with a distinct growth-differentiating effect; epithelial growth is enhanced and fibroblasts are inhibited.[2,3] Six such cover slips with the plastic membrane facing upward are placed on a specially prepared glass slide. Small tissue explants are placed on each of the 6 plastic membranes. Special nutrient fluid (plasma-extract supernate, embryonic extract and Tyrode) is added to each culture and maintained in place by a small glass ring sealed with vaseline. The 6 cover slips with their membranes and cultures are covered with a petri dish and infected at the start or after growth has taken place. The nutrient medium is changed at 3-4 day intervals if indicated. Strict asepsis is observed throughout the tissue culture procedure. At an appropriate time, usually 3-5 days for vaccinia, the cover slips with the plastic membrane to which are now attached the growing tissue cells are lifted from the culture chamber. A cover slip with the plastic surface facing up is placed in a petri dish containing Ringer's solution; under these conditions the membrane detaches itself completely from the cover slip and floats on the surface. The floating membrane in the petri dish is examined under low power with an ordinary microscope and the desired fields are selected. These are fixed in the center of the metallic mount by a modification[4] of the usual technic employed in electronmicroscopy.

In the present work, minute explants (weighing about 0.1 mg) prepared from the renal cortex of adult rabbits were used. Cultures were inoculated with a washed suspension of neurovaccinia elementary bodies. Elec-

1 Richards, A. G., Jr., Anderson, T. F., and Hance, R. T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 148.

2 Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, **81**, 233.

3 Wirth, J., and Barski, G., *Ann. Inst. Pasteur*, 1947, **73**, 987.

4 Wirth, J., *C. R. Acad. Sci.*, 1947, **225**, 899.

5 Wirth, J., in: Levaditi, C., and Lépine, P., *Traité des Ultravirus des Maladies Humaines*, 2nd edition, Paris, 1948 (Maloine éd.), page 1683.

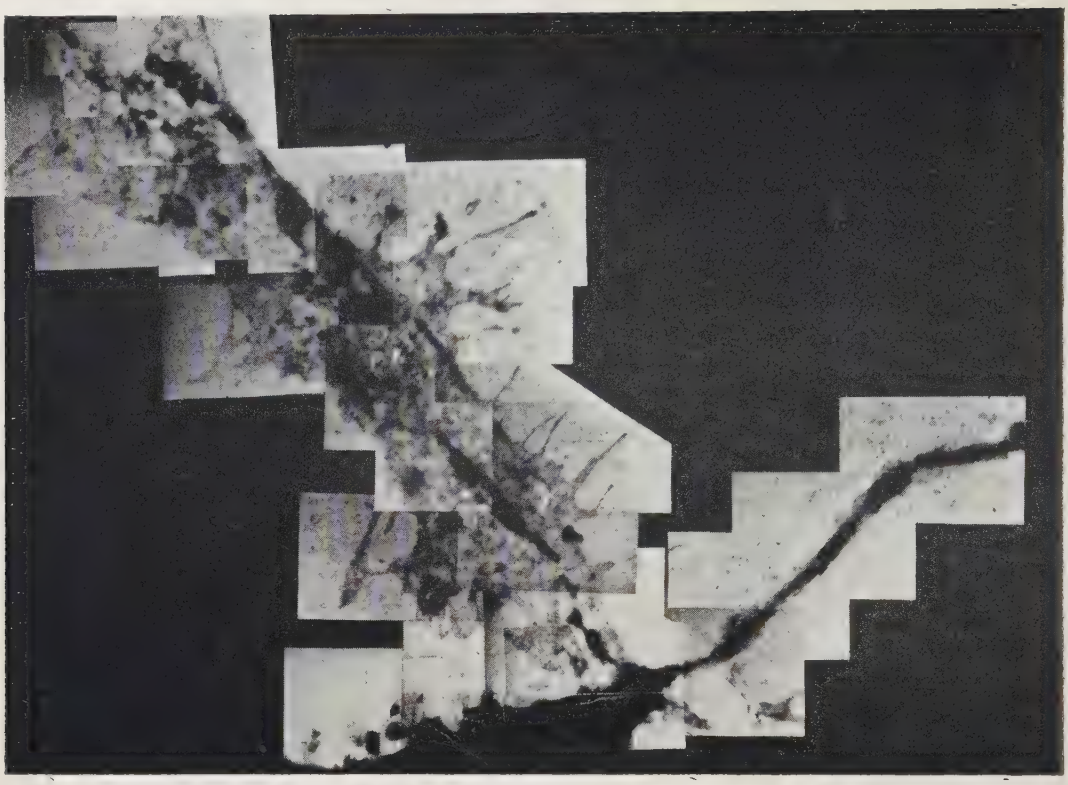

FIG. 1.
Composite electron micrograph of a cell infected with vaccinia. Elementary body-like structures are seen in upper left portion of picture.

FIG. 2.
Electron micrograph representing a portion of the cell containing numerous elementary body-like structures.

tron micrographs were taken with the C.S.F. electrostatic electron microscope.

Results. Cultures of explants from kidneys of adult rabbits grown by the method described provide essentially pure growth of epithelial cells in a single layer. The technic presents several advantages over the technics previously employed: (1) It provides a rela-

tively simple means for maintaining true cultures a number of days or weeks[6] without transfer; (2) the method does not require the use of a plasma coagulum (fibrin being eliminated); and (3) a monolayer of living cells, almost entirely epithelial, can be obtained for special studies.

A topographical survey of a single cell infected with vaccinia is illustrated in Fig. 1. The individual electron micrographs which make up the composite picture were taken at a magnification of 21,000; after assembly the composite picture was reduced somewhat. The opaque spherical structures occurring in the cytoplasm at the upper left corner are interpreted as elementary bodies of vaccinia seen individually and in clusters. Fig. 2 reproduces one of the micrographs containing the elementary body-like structures which make up Fig. 1. This electron micrograph was also taken at a magnification of 21,000 but has not been reduced. An idea of the reduction in size of the composite picture is obtained by comparing Fig. 2 with the individual components of Fig. 1. Spherical opaque structures such as those observed in Fig. 1 and 2 are regarded as elementary bodies since they have not been observed in similar preparations of uninfected rabbit kidney tissue culture cells. The present method adds another approach to the study of the intracellular growth of vaccine virus which has in the past been fruitfully investigated by microscopic technics employing visible light.[7,8]

Summary. A technic has been devised which provides true cultures of mammalian cells for electronmicroscopy. The usefulness of this procedure in the study of viruses is indicated.

[6] Barski, G., *Ann. Inst. Pasteur,* 1948, **74**, 312.

[7] Nauck, E. G., and Robinow, C., *Zentralbl. f. Bakt.* (Abt. 1), 1936, **135**, 437.

[8] Bland, J. O. W., and Robinow, C. F., *J. Path. and Bact.,* 1939, **48**, 381.

16824

Adrenalectomy and Pituitary Adrenocorticotrophic Hormone Content.*†

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Alterations in the weight and chemical composition of the adrenal gland have been used as quantitative indices for measurement of the rate of discharge of adrenocorticotrophic hormone (ACTH) from the adenohypophysis following application of stress.[1] These indices have been called "target gland indices". It is now possible to complement our knowledge of the pituitary-adrenal system gained from such indices with data on adenohypophyseal content of ACTH. The present report is one of several studies concerned with factors regulating the synthesis and discharge of pituitary ACTH, and deals especially with the changes following adrenalectomy in the rat.

Methods. Male rats from the Sprague-Dawley farm were used. Twenty rats, 230-275 g in body weight, were employed in two experiments conducted at different times. In Experiment I, 6 rats were bilaterally adrenalectomized and 4 rats served as controls; in Experiment II, 4 rats were bilaterally adrenalectomized, 3 rats were subjected to sham adrenalectomy and 3 rats served as controls.

* Supported by a grant from the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

† With the statistical assistance of Marion A. Sayers.

‡ Fellow of the American Bureau for Medical Aid to China.

[1] Sayers, G., and Sayers, M. A., *Recent Progress in Hormone Research,* 1948, **2**, 81.

TABLE I.
Pituitary Adrenocorticotrophic Hormone Potency after Adrenalectomy.

Expt.	Donors	Dose of pituitary, μg per 100 g of recipient rats	Reduction of adrenal ascorbic acid content of recipient rat mg per 100 g adrenal tissue
I	Control (4)*	40	146,† 173, 144
		10	84, 94, 59
	Adrenex (6)	40	76, 12, 28
		10	14, 0, 17
II	Control (3)	40	180, 173, 118, 103
		10	68, 26, 18
	Sham (3)	40	150, 117, 112, 125
		10	95, 63, 18, 15
	Adrenex (4)	160	148, 107, 141, 126
		40	64, 25, 54, 81
III	Control (13)	40	147, 189, 147
		20	157, 120, 140
		10	111, 116, 63
		5	97, 26, 26
	Scald (6)	40	158, 133, 151, 147, 174
		20	88, 107
		10	69, 70, 73, 46

* No. of rats in group.
† Each value in right-hand column represents result in one recipient rat.

Twenty-four hours after removal of the adrenals, all rats in the various groups were anesthetized with sodium pentobarbital and the pituitaries removed. During the 24-hour period the animals were fasted but had free access to tap water. A third experiment (III) was conducted in order to compare the changes produced by adrenalectomy with those produced by a severe stress. Nineteen rats weighing 270 to 350 g were fasted 24 hours; 6 were then scalded and the remaining 13 served as controls. All were sacrificed after an additional 24-hour period of fasting. For scalding the rats were immersed up to the neck for 7 seconds in an aqueous detergent ("Duponal") solution at 71 ± 1°C.

The anterior lobes were carefully freed of posterior pituitary tissue, frozen and lyophilized. The pituitaries of each group of rats were pooled, and ground to a homogeneous powder. Aliquots of this powder weighing from about one-half to two mg were extracted successively with 3 portions of a solution of 0.9% NaCl made alkaline to 0.01 N with NaOH. The total volume of extraction fluid was adjusted according to the potency of the tissue and varied from 0.5 ml per 40 μg to 0.5 ml per 160 μg of dry tissue. Appropriate dilutions were prepared for the various dose levels. The extracts were injected into hypophysectomized recipient rats (0.5 ml per 100 g of body weight) and the degree of reduction of adrenal ascorbic acid was measured by the difference between the concentration of ascorbic acid in the left (control) adrenal removed immediately before administration of the pituitary extract and that of the right adrenal removed an hour after such administration.[2]

Results. The assay data from the three experiments are presented in Table I. The ability of extracts of pituitaries to deplete the adrenal ascorbic acid of hypophysectomized recipient rats is a measure of their content of ACTH. It is apparent that the content of ACTH in the adenohypophyses of adrenalectomized rats is markedly reduced. In contrast, the scalded animals exhibit only a slight reduction in ACTH content of their pituitaries.

The results of the 3 experiments have been combined for statistical analysis in Table II. Since 10 μg of pituitary tissue from adrenalectomized animals (Expt. I) failed to give a significant response, the results obtained with this dose have not been included in the analy-

[2] Sayers, M. A., Sayers, G., and Woodbury, L. A., *Endocrinology*, 1948, **42**, 379.

TABLE II.
Relative Content of ACTH in Rat Adenohypophyses.

Groups compared	No. recipient rats	b	s	λ	M	Antilog of M	S_M	Antilog (M—S_M)-antilog (M+S_M)
Sham (3)*	8	130	29.7	0.228				
					$\bar{1}.861$	0.73	±0.097	0.58-0.91
Control A (7)	13	149	30.0	0.201				
Adrenex (10)	11	136	24.4	0.180				
					$\bar{1}.306$	0.20	±0.083	0.17-0.25
Control A (7)	13	149	30.0	0.201				
24-hour scald (6)	11	147	13.9	0.095				
					$\bar{1}.814$	0.65	±0.069	0.56-0.76
Control B (13)	12	125	27.3	0.218				

b = slope of the log dose response curve; s = standard deviation of all of the individual responses about this curve (a straight line fitted by the method of least squares); λ = s/b and is a measure of the accuracy of the assay method; M = logarithm of the ratio of the potencies; S_M = standard error of M; antilog (M—S_M)-antilog (M+S_M) is the range of estimate for one std. error.

* No. rats in donor groups in parentheses.

sis. The 7 control rats of Expt. I and II were combined as "Control A" in Table II. The 13 control rats of the scald experiment (III) serve as "Control B" in Table II. The analyses depicted in Table II include 3 assay pairs, as follows: sham-operated rats compared with Control A; adrenalectomized rats compared with Control A; scalded rats compared with Control B. No significant difference exists between the slopes of the log dose-response curves (b-values) of any two groups compared, a fact which indicates that the observed alterations in biological activity are quantitative and not qualitative. Antilog M, the ratio of the adrenocorticotrophic hormone potencies for each assay pair, is an expression of the relative content of ACTH in the pituitaries of the experimental as compared with the control groups. A slight reduction (27%) of the ACTH content of the pituitary took place in the sham-operated animals. The effects of the scald, acting as a continuous stress over a 24-hour period, reduced the content of ACTH in the adenohypophysis by only 35%. In contrast, adrenalectomy reduced the ACTH content by 80%. The differences in ACTH content cannot be accounted for by differences in the weights of the pituitaries. The mean dry weights of the pituitaries (expressed as mg per rat) were as follows: control A, 0.88; sham-operated, 0.58; adrenalectomized, 0.77; control B, 1.21; scalded, 1.35.

Preliminary histological studies employing the staining technic of Koneff[3] have revealed no striking cytological changes in the anterior pituitary of adrenalectomized rats associated with the marked reduction in ACTH content.

Discussion. In an attempt to elucidate the factors responsible for a reduction in the ACTH content of the pituitary following stress we have formulated the problem in its simplest terms, by assuming that two processes, synthesis (S) and discharge (D) of ACTH, can each separately undergo no change in rate ($_n$), an increase in rate (↑) or a decrease in rate (↓). These 6 variables give rise, theoretically to 9 combinations, represented symbolically as S_nD_n, $S_nD\uparrow$, $S_nD\downarrow$, $S\uparrow D_n$, $S\uparrow D\uparrow$, $S\uparrow D\downarrow$, $S\downarrow D_n$, $S\downarrow D\uparrow$ and $S\downarrow D\downarrow$. Four of these combinations S_nD_n, $S_nD\downarrow$, $S\uparrow D_n$ and $S\uparrow D\downarrow$, are eliminated at once because the problem at hand concerns only a reduction in content of ACTH.

It is possible to rule out 2 more combinations, $S\downarrow D_n$ and $S\downarrow D\downarrow$, on the basis of the fact that they cannot account for the increased rate of discharge of ACTH always associated with stress; studies in this laboratory[1] have shown that a discharge of ACTH follows the subjection of an animal to any of a variety of stresses, such as sham adrenalectomy or scald. Of the remaining 3 combinations, $S_nD\uparrow$, $S\uparrow D\uparrow$ and $S\downarrow D\uparrow$, one does

[3] Koneff, A. A., *Stain Technol.*, 1938, **13**, 49.

not seem probable, namely, S↓D↑, because it appears most unlikely that the rate of synthesis of ACTH would be depressed when there is an increased body need for the hormone. Thus only two combinations remain to be considered as possibilities, namely, (1) increased discharge of ACTH associated with no change in the rate of synthesis, and (2) increased discharge associated with an increased rate of synthesis.

It is likely that in mild stress the first possibility obtains; in severe stress, the second possibility. The pituitary stores a relatively large quantity of ACTH; there is an amount of ACTH in one rat pituitary sufficient to produce a marked reduction (50% of maximal) in the ascorbic acid content of the adrenals of 100 rats. Mild stress produces a reduction in adrenal ascorbic acid content which is approximately 50% of maximum. Therefore, the quantity of ACTH normally stored in the pituitary is 100-fold greater than the amount required to meet the immediate needs of the organism subjected to mild, short-acting forms of stress; synthesis of new hormone would not be needed in order to cope with the stressful situation. However, in the case of severe, long-acting forms of stress, for example, scald, synthesis must be accelerated so that the store of ACTH can be fairly well maintained despite its rapid and continuous discharge. In Table II, it can be seen that the ACTH content of scalded rats was maintained at 65% of the control level.

In contrast, adrenalectomy is followed by a very marked reduction in the pituitary content of ACTH. Whereas the severe continuous stress of scalding resulted in a reduction of only 35%, adrenalectomy led to a reduction of 80% in hormone content. Unfortunately, direct experimental evidence regarding rate of discharge of ACTH, such as that available in the stress experiments, was not obtainable in the adrenalectomy experiments, because adrenalectomized animals lack the target gland which provides a simple means for measuring rate of ACTH discharge. Therefore, one must consider depression of synthesis as well as increased rate of discharge as a factor responsible for the depletion of stores of ACTH in the pituitary 24 hours after adrenalectomy. However, we are inclined to favor the view that the rate of discharge of ACTH is at a maximum when there is a complete absence of the target gland secretion. This latter possibility is in keeping with other experimental data from this laboratory[4] which have been interpreted to mean that the rate of discharge of ACTH increases as the level of cortical hormones in the body fluids falls. According to this concept, adrenalectomy provides a maximum stimulus for discharge of ACTH since it produces the lowest possible titers of cortical steroids in body fluids. Determination of body fluid titers of ACTH in adrenalectomized animals by direct assay would be of considerable help in assessing the relative roles of synthesis and discharge as factors regulating the ACTH stores of the pituitary.

Summary. Twenty-four hours after adrenalectomy the content of ACTH in the pituitary of the rat is reduced by 80%. Sham adrenalectomy and scalding reduce the content of ACTH by only 27% and 35%, respectively. The significance of these observations for the problem of the regulation of rate of synthesis and discharge of pituitary ACTH is discussed.

The authors are indebted to Dr. Louis S. Goodman and Dr. Walter S. Loewe for their many helpful suggestions and criticisms during the preparation of the manuscript.

[4] Sayers, G., and Sayers, M. A., *Endocrinology*, 1947, **40**, 265.

16825

Effect of Injecting Crystalline Tetanal Toxin and Tetanal Antitoxin Into Mice.

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Pillemer and Wartman[1] have shown that the administration of crystalline tetanal toxin in single doses ranging from 0.25 to 500,000 M.L.D. produced no pathological lesions in the muscles or central nervous system of white Swiss mice. Similar results were obtained upon the injection of numerous sublethal doses of toxin. Experiments were subsequently undertaken to determine whether the administration of tetanal antitoxin would produce any pathological lesions in mice that had received various amounts of crystalline toxin. This seemed important because many of the patients with tetanus on whom autopsy was performed have received antitoxin.

Experiments. White Swiss inbred mice, which weighed between 15 and 20 g, were divided into 8 groups. Mice in Groups I to IV were given 1 M.L.D. of crystalline tetanal toxin followed by 25 units of tetanal antitoxin. In Group I the antitoxin was administered one day after the toxin; in Group II 2 days afterwards; in Group III 3 days afterwards; and in Group IV 4 days afterwards. Mice in Group V were given 0.5 M.L.D. of toxin followed 5 days later by 25 units of antitoxin. In Group VI the procedure was reversed and the mice were given first 25 units of antitoxin and then 1 M.L.D. of toxin every day for 6 successive days, receiving in all 6 M.L.D. of toxin. Animals in Group VII were given 25 units of antitoxin, and those in Group VIII 2500 units of antitoxin. None of the mice in Groups VII and VIII received tetanal toxin.

Crystalline tetanal toxin[2,3] and a highly purified antitoxin (Lederle) were used in all experiments. The toxin was dissolved in 0.3 M glycine and injected into the gluteal muscles at the base of the tail on the right side. The antitoxin was diluted in 0.9% saline and injected intraperitoneally. The mice were amply fed and allowed as much water as they would drink.

The mice were killed at the desired intervals with ether, except those in Groups III and IV which died. Autopsies were performed immediately after death, and tissues were fixed for 24 hours in 10% formalin and Zenker's fluid. Sections were stained with hemalum and aqueous eosin and, in addition, the brain, spinal cord, and peripheral nerves were stained specifically for Nissl substance and myelin. Sections were made of the following organs: the anterior, middle, and posterior thirds of the brain; the lumbar, thoracic, and cervical portions of the spinal cord; anterior and posterior nerve roots; sciatic nerve; the cerebral and spinal meninges; the skeletal muscles from both the injected and the opposite side of the tail, both gastrocnemius muscles, and the muscles along the vertebral column.

Results. When 25 units of tetanal antitoxin were given to white Swiss mice within 24 hours after the intramuscular injection of 1 M.L.D. of crystalline tetanus toxin, the animals were protected from the development of clinical tetanus. When the antitoxin was not given until later, the signs of classical tetanus developed and the mice died. No detectable pathological changes occurred in animals which survived for as long as 14 days. If only 0.5 M.L.D. of toxin was injected, the administration of 25 units of antitoxin was effective even if delayed for several days. When given as long as 5 days after the toxin, some of the mice developed

1 Pillemer, L., and Wartman, W. B., *J. Immunol.*, 1947, **55**, 277.

2 Pillemer, L., Wittler, R. G., and Grossberg, D. B., *Science*, 1946, **103**, 615.

3 Pillemer, L., Wittler, R. G., Burrell, J. I., and Grossberg, D. B., *J. Exp. Med.*, 1948, **88**, 205.

tetanus, but the signs were only mild to moderate. In these mice, also, no significant pathological lesions were discovered.

The intraperitoneal injection of either 25 or 2500 units of tetanal antitoxin caused no recognizable clinical or pathological manifestations.

Summary. The administration of 25 units of tetanal antitoxin to white Swiss mice which had previously received 1 M.L.D. of crystalline tetanal toxin, produced no discoverable lesions in the central nervous system, peripheral nerves, or skeletal muscles, irrespective of whether the animals developed clinical tetanus, or of how long they survived. Likewise, the intraperitoneal injection of either 25 or 2500 units of tetanal antitoxin caused no changes which could be detected by ordinary histopathological technics.

16826

Effect of *Lithospermum ruderale* on the Gonadotropic Potency of the Pituitary Gland.*

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(Introduced by Raymond N. Bieter.)

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The crude plant, *Lithospermum ruderale,* has been shown to cause prolonged or persistent diestrous in mice with previously regular estrous cycles.[1,2] Since the administration of either follicle-stimulating hormone or estrogenic hormone induces estrus in animals under treatment with Lithosperm and since Lithosperm causes a decrease in the weights of pituitary glands the suggestion has been made that the drug acts on the pituitary gland to decrease the formation or secretion of gonadotropic hormone.[1] The present paper deals with the biological assay for gonadotropic potency of pituitary glands from treated and control animals.

Experimental. Female mice, 21 days old, were divided into 3 groups, distributing littermates between the groups as evenly as possible. Group I called *Lithosperm donors,* consisted of 9 mice, each of which was given an injection of two pituitary glands from donor mice which had been on a 20% ground Lithosperm diet for one week or more. In Group II, the control donors, each of 9 mice received an injection of 2 pituitary glands from donor mice on control diet, Purina fox chow. Group III served as uninjected controls, receiving no pituitary injections. Donor mice for Groups I and II were adult females and were sacrificed during diestrus only. To make the pituitary injections a method similar to that of Smith and Engle[3] was used. Donor mice were killed with ether, the skin on back of head calvarium and brain removed and pituitary gland lifted out. Recipient mice were lightly anesthetized with ether and the pituitary glands placed through a small incision into the thigh muscles. Mice were autopsied at 26 days of age, *i.e.* 5 days after injection. The ovaries, uterus plus vagina and adrenal glands were dissected out and weighed in the moist state.

The data obtained are presented in Table I. Evidence of gonadotropic stimulation was

* This work constitutes part of a study supported by a grant from the U. S. Public Health Service.

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3 Smith, P. E., and Engle, E. T., *Am. J. Anat.,* 1927, **40**, 159.

TABLE I.
Effect of Injections of Pituitary Glands Obtained from Lithosperm and Control Donors on Organs of 21-day-old Mice.

Group	No. mice	Organ weight in mg			Body weight in g	
		Ovaries	Uterus + vagina	Adrenals	21 days old	26 days old
I. Lith. donors	9	5.0 ± 0.3	14.6 ± 1.1	2.4 ± 0.2	6.9 ± 0.5	9.0 ± 0.7
II. Control donors	9	5.8 ± 0.4	26.2 ± 4.0	2.7 ± 0.2	6.9 ± 0.2	9.5 ± 0.3
III. Uninjected controls	10	4.8 ± 0.3	13.5 ± 1.7	1.9 ± 0.2	6.9 ± 0.6	8.6 ± 0.9

not seen in mice receiving pituitary injections from Lithosperm donors (Group I) inasmuch as there is no significant difference between the weights of the uterus plus vagina in these mice and in the uninjected controls (Group III). On the other hand considerable gonadotropic stimulation was evident in the mice receiving pituitary injections from control donors (Group II), the uterus plus vagina weighing 95% more than that of the uninjected controls and 79% more than that of the Lithosperm donor. These differences are significant statistically. Thus the results show that the gonadotropic potency of pituitary glands of adult female mice receiving Lithosperm was decidedly less than that of similar control mice.

No significant differences in the weights of ovaries or adrenal glands or in body weight between the 3 groups were obtained. Had the mice been injected with more pituitary glands, some differences might have been noted, at least in ovarian weight. Increase in the weight of ovaries is also an indicator of gonadotropic stimulation, but a less sensitive one than that of the uterus.[4]

The concentration of the sample of Lithosperm (20%) used in this experiment is a threshold one, producing continuous diestrous in 55 per cent of mice of a sensitive strain (C_3H). A concentration of 30% is required to produce continuous diestrous in all such mice.

Discussion. The finding that Lithosperm decreases the gonadotropic potency of the pituitary gland aids in the understanding of the mechanism of action of this crude drug. The contention that the action is not a direct one on the vagina, ovaries or circulating gonadotropin but involves rather the gonadotropic activity of the pituitary gland is strengthened. Zahl[5] has shown that no histological changes are evident in the pituitary gland after prolonged Lithosperm administration and that normal estrous cycles are reestablished quickly after discontinuance of the drug, even after 8 months of therapy. Thus it appears that Lithosperm inhibits in some way the formation of gonadotropic hormone without producing irreversible or histological damage to the pituitary gland.

Conclusion. The gonadotropic potency of pituitary glands of adult female mice is decreased by the administration of *Lithospermum ruderale* as evidenced by a lack of increase in weight of the uterus plus vagina in 21-day-old mice which have been injected with pituitary glands from treated mice.

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16827

Thymic Atrophy and Lymphoid Hyperplasia in Mice Bearing Sarcoma 180.

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The changes in the size or weight of the thymus induced by various endocrine factors have been assumed to parallel those occurring in lymph nodes.[1-4] This is instanced by the concurrent hypertrophy of these tissues following adrenalectomy[5,6] and the concurrent atrophy after thyroidectomy,[7,8] gonadectomy,[7,9] the administration of cortical hormones[10-12] and pituitary adrenocorticotrophin.[13-15] While this evidence strongly supports the contention that the thymus acts as part of the lymphoid tissue, opinions to the contrary are widely held[16-18] and have recently been reviewed.[19]

One of us recently described[20] the *pronounced hyperplasia* of, and the increased nitrogen deposition in lymphoid tissue (as represented by the combined thymus and lymph node weight and nitrogen content) of male mice of the CFW strain bearing implants of Sarcoma 180. Independently, the other of us observed that Sarcoma 180 in female mice of the same strain evoked a *significant atrophy* of the thymus.[21] It thus appeared that under the conditions of these experiments identical factors could produce a *simultaneous thymic atrophy and lymphatic hyperplasia.* The present study demonstrates this fact.

Experimental. Sarcoma 180 was implanted subcutaneously near the axilla[22] as previously described[20,21] into 12 male and 12 female CFW mice (Carworth Farm) of approximately 18-20 g body weight; an equal number of untreated mice of both sexes served as controls. The latter group (6 animals per cage) received an amount of Purina Fox Chow equivalent to that consumed by the corresponding tumor bearing group and all animals were given tap water *ad libitum;* this control of food intake was later found to have no apparent effect on the tissue changes observed.

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11 Ingle, D. J., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 174.

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TABLE I. Weight Changes of Thymus and Lymph Nodes in Intact Mice Bearing Sarcoma 180.

Sex	Group	No. animals	Final body wt, g	Thymus wt		Lymph nodes wt	
				mg	mg/100 g B.W.	mg	mg/100 g B.W.
Male	Tumor bearing	10	16.1 ± 0.5	9.8 ± 1.9	61 ± 10.5	54.4 ± 5.4	338 ± 28.9
	Controls	12	18.1 ± 0.5	27.0 ± 2.7	149 ± 12.5	30.5 ± 2.7	169 ± 13.3
Female	Tumor bearing	10	16.0 ± 0.5	16.4 ± 2.5	102 ± 14.0	63.6 ± 0.4	398 ± 19.4
	Controls	12	17.6 ± 0.5	30.9 ± 2.4	176 ± 12.9	34.8 ± 0.4	198 ± 20.3

* Mean ± standard error.

TABLE II. Weight Changes of Thymus and Lymph Nodes in Hypophysectomized Mice Bearing Sarcoma 180.

Sex	Group	No. animals	Final body wt, g	Thymus wt		Lymph nodes wt	
				mg	mg/100 g B.W.	mg	mg/100 g B.W.
Female	Tumor bearing	7	14.8 ± 1.2	26.1 ± 1.9	176 ± 12.5	65.9 ± 2.5	445 ± 17.1
	Controls	10	14.4 ± 0.3	36.4 ± 2.2	252 ± 14.3	45.0 ± 3.1	312 ± 22.8

Twelve days following tumor implantation, all animals were sacrificed by cervical fracture following a 16 hour fast, during which water remained available. The thymus and 3 lymph nodes (one axillary node from each side and one mesenteric node) were dissected from each animal and immediately weighed separately; other tissues were removed from these mice for additional studies which will be reported later. The weight of thymus and the combined weights of the three nodes, expressed in mg of fresh tissue and as mg per 100 g of final body weight are given in Table I.

In a second experiment, female mice of the same strain, weighing 12 to 14 g were hypophysectomized. Five days later Sarcoma 180 was implanted in the usual way in one half of the animals, the remainder serving as controls. From the time of pituitary removal the mice were maintained as described above except for the replacement of drinking water by physiological saline solution. The animals were allowed to survive for ten days following implantation and during this time one half of the tumor bearing group had died, while only 2 of the hypophysectomized control group were lost. Both groups were then sacrificed and the tissues dissected and weighed as described above. The completeness of pituitary removal, already indicated by the cessation of growth over the experimental period, was verified by histological examination of the base of the skull. Table II lists the weights of the thymus and the combined weights of the 3 lymph nodes of the hypophysectomized animals.

Discussion. The data shown in Table I and Fig. 1 demonstrate that mice of the CFW strain, bearing Sarcoma 180, exhibit atrophy of the thymus averaging 50% by weight, and an increase in lymph node weight of the order of 100%. No important sex difference was noted. Visual inspection indicated that the weight increase in the selected lymph nodes reflected a generalized change in the entire lymphatic system of the tumor bearing mice, a fact which has since been confirmed.[23] The

[23] Savard, K., and Tompkins, M., unpublished data.

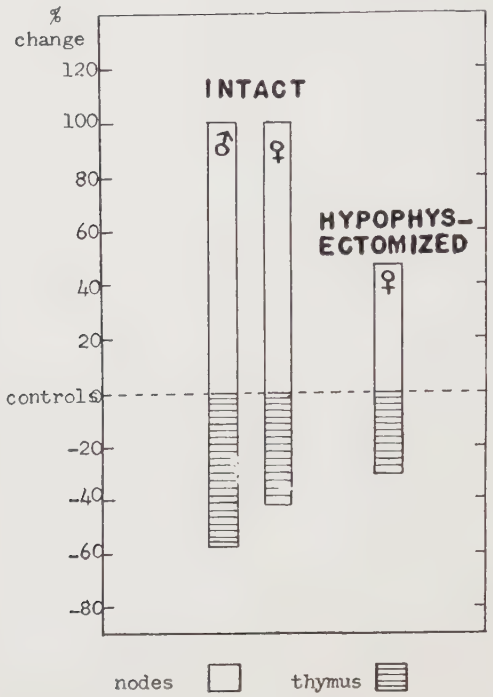

FIG. 1.

Per cent change over control values of thymus and lymph nodes weights expressed as mg per 100 g of body weight. The columns on the left refer to the intact animals; the one on the right refers to the hypophysectomized mice.

possibility of the hyperplasia being due to infection, regional lymphadenopathy or metastases of the tumor has been excluded in an earlier series by histological studies and a true lymphatic hyperplasia established by the clear cut increase in nitrogen content of these nodes.[20] There was thus, under the conditions of the experiment, a distinct dissociation of the response of the thymus and of the lymph nodes to the presence of growing Sarcoma 180.

In view of the fact that the growth of Sarcoma 180 in the CFW strain of mice is accompanied by adrenal changes indicative of an increased stimulation of the latter organ by the anterior pituitary[21] the possibility of a relationship between the endocrine system and the lymphatic and thymic changes was considered. The results of the second experiment shown in Table II indicate however that in the hypophysectomized animal, the growing tumor evoked a thymic atrophy and lymphatic hyperplasia, though to a lesser degree than in the intact animal; the adrenal hypertrophy and lowered ascorbic acid levels induced by the tumor in the intact animal[21] were, on the other hand, not observed in the hypophysectomized mice.[23] It is thus implied that the thymic and lymphatic changes are not mediated through the pituitary-adrenal system.

Summary. Transplanted Sarcoma 180 after 12 days of growth evokes thymic atrophy and lymphatic hyperplasia in mice of the CFW strain. These changes are not prevented by hypophysectomy, thereby excluding pituitary-adrenal mediation.

The authors wish to thank Dr. George Woolley and Miss Rosann Chute for the preparation of the hypophysectomized mice, and Miss Mary Tompkins and Miss Iris Forbes for their technical assistance.

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Response of Guinea Pigs to Diets Deficient in Choline.

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The hepatic choline concentration of the choline-deficient rat, when calculated on a fat-free basis, is not lower than that of normal rats.[1,2] This fact has been correlated with the observation that the choline oxidase activity of the fatty livers of choline deficient rats is markedly suppressed[3] and the suggestion advanced that it is the diminished choline

[1] Jacobi, H. P., and Baumann, C. A., *J. Biol. Chem.*, 1942, **142**, 65.

[2] Handler, P., and Dann, W. J., *J. Biol. Chem.*, 1942, **146**, 357.

[3] Handler, P., and Bernheim, F., *J. Biol. Chem.*, 1942, **144**, 401.

TABLE I.
Composition of Diets.

	1	2	3	4	5	6	7
Casein	12	12	12	12	10	6	6
Cellulose	20	20	10	20	20	15	10
Salts[18]	4	4	4	5	5	5	5
Liver "L"	3	3	3	5	5	5	4
Lard	10	10	15		20	15	25
Glucose	48	48	52	55			
Sucrose					23	21	15
Cod liver oil	3	3	3	1			
Cystine	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Cholesterol	0.3	0.3	0.3	0.3	0.5	0.5	0.5
Inositol	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Glycocyamine	0.1	0.3	0.3	0.3	0.5	0.5	0.3
Arginine				1.0	1.0	1.0	0.5
Glycine				0.3	0.3	0.3	0.3
Peanut Meal						30	30
Cerophyl							3

oxidase activity which permits the existence of a normal hepatic choline concentration (as phospholipid) despite the dietary choline deficiency.[1,3] Choline deficiency and its manifestations have been most frequently studied in the rat[4] but have also been observed in the dog,[5] mouse,[6] and chick[7] as well as implicated in the etiology of hepatic cirrhosis in man. The livers of these species have all been shown to possess marked choline oxidase activity.[8] Although fatty livers have frequently been observed in the guinea pig, under various conditions there has been, as yet, no demonstration that these have been, in any case, the result of dietary choline deficiency. Since the liver of this species is unique among common laboratory animals in that it possesses little or no choline oxidase activity,[8] it was of considerable interest to determine the susceptibility of this species to dietary choline deficiency. The present paper describes a number of attempts to elicit some manifestation of choline deficiency in young guinea pigs under conditions which were quite effective for the rat.

Experimental. In preliminary studies various procedures for the feeding of synthetic rations to young guinea pigs were investigated. In some instances the newborn pigs were offered the experimental diets while still suckling and were continued on them after separation from the sow. A few attempts were made to offer such rations to one-week-old pigs weaned at that time. However, the data to be presented were all obtained in the following manner. Weanling guinea pigs were immediately offered a diet consisting of equal parts of the synthetic ration and minced carrots. After 4 days the amount of carrots was reduced by half and 4 days later the carrots were removed entirely. A group of rats, as controls, was also offered each of the experimental rations in similar fashion. All animals were housed in individual cages with food and water available *ad libitum*. Three weeks after deletion of the carrots from the diet the animals were sacrificed by decapitation and their livers taken for lipid analysis.[9]

Using this feeding technic relatively few young pigs refused the experimental rations. All animals which failed to eat adequately or which lost weight unduly and died before termination of the feeding trial were discounted in evaluating the results of these experiments. This was considered valid since approximately equal numbers of pigs died on both choline-deficient and choline-supplemented rations. It is of interest that, in many instances, on each of the experimental rations employed, pigs died suddenly after a

4 Best, C. H., and Lucas, C. C., *Vitamins and Hormones*, 1943, **1**, 1.

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TABLE II.
Liver Lipids of Guinea Pigs and Rats on Choline-Deficient Diets.

Group	Species	No. animals	Diet	Initial wt, g	Wt change, g	Food intake, g/day	Liver lipids, % wet wt
1	GP	6	1	188	— 7	9.3	4.8
2	”	5	1 + choline	181	— 6	10.0	4.6
101	Rat	6	1	82	29	8.3	11.6
102	”	6	1 + ”	76	31	8.1	5.3
3	GP	5	2	189	2	9.2	4.4
4	”	6	2 + ”	196	— 3	9.6	4.1
103	Rat	5	2	68	26	7.7	13.8
104	”	5	2 + ”	73	23	7.9	6.1
5	GP	7	3	161	—11	9.1	4.3
6	”	6	3 + ”	173	— 6	9.9	4.4
105	Rat	6	3	84	16	7.2	15.6
106	”	6	3 + ”	76	24	7.9	5.9
7	GP	5	4	143	9	8.7	4.9
8	”	8	4 + ”	148	4	8.4	5.2
107	Rat	6	4	61	29	8.0	10.8
108	”	6	4 + ”	60	27	8.0	4.7
9	GP	6	5	148	—13	6.9	5.6
10	”	6	5 + ”	156	—17	7.1	5.3
109	Rat	5	5	69	17	6.3	21.7
110	”	5	5 + ”	64	23	6.1	6.3
11	GP	7	6	173	41	12.7	7.4
12	”	6	6 + ”	184	37	11.9	7.7
111	Rat	6	6	67	16	6.7	24.2
112	”	6	6 + ”	72	23	6.6	5.8
13	GP	6	7	177	47	11.8	8.3
14	”	6	7 + ”	189	41	11.4	6.9
15	”	5	7 + methionine	180	40	11.5	8.2
113	Rat	5	7	74	14	7.0	26.7
114	”	5	7 + choline	61	19	6.9	6.8

variable period of reasonable weight gain or maintenance and apparently adequate food consumption. Similar deaths have not been observed in animals living on the stock diets commonly employed for this species.

The various diets employed are summarized in Table I. Each diet was offered to a group of control rats as well as to guinea pigs. To each kilo of each diet was added thiamine 10 mg, riboflavin 12 mg, pyridoxine 10 mg, *p*-aminobenzoic acid 25 mg, calcium pantothenate 50 mg, niacin 50 mg, ascorbic acid 200 mg, α-tocopherol 100 mg, and 2-methyl-1, 4-naphthoquinone 10 mg. All animals received 50 γ biotin and 50 γ folic acid 3 times weekly by pipette. All animals not receiving cod liver oil in the diet were given 2 drops of percomorph oil 3 times weekly. All animals on diets 3 - 7 were given 1 drop of cream 3 times weekly by pipette. Not indicated in the tables is the fact that each of these diets was also fed without inositol. However, in no case did the absence of this substance appear to affect the results. Choline, when present was incorporated as 0.6% of the diet as was DL-methionine. The results are summarized in Table II.

The inclusion of liver fraction “L” in all diets was based on the findings of Woolley and Sprince[10] and has been found to contain but 0.87 mg of choline per gram. Arginine and glycine were added to diets 4 - 7 as these have been found to improve the growth of guinea pigs on low protein rations.[11] Glycocyamine was added to diets 2 - 7 since, like nicotinamide, it forces a demand for methyl groups

[10] Woolley, D. W., and Sprince, H., *J. Biol. Chem.*, 1944, **153**, 687.

[11] Woolley, D. W. personal communication.

and increases the choline and/or methionine requirement of the rat. [12] "Cerophyl" was included in diet 7 as a source of "grass juice factor". The rations containing alcohol-extracted peanut meal were patterned after those used by Engel and Salmon[13] and proved to be the most successful diets in this study. It will be seen that most of the guinea pigs on diets 1 - 5 grew very poorly or not at all and a considerable number actually lost weight during the experimental period. It has been noted that under such conditions little or no fat accumulates in rat livers despite choline deficiency[2,14,15] and it is now well recognized that the optimal conditions for eliciting the hepatic and renal manifestations of choline deficiency are those which permit the maximum growth rate compatible with the relatively low protein diets necessary in such studies.[16] The peanut meal rations 6 and 7 much more satisfactorily met these requirements as seen in Table II.

It is apparent that while diets 1 - 5 were sufficiently lacking in choline and methionine to produce fatty livers in rats, they did not result in the accumulation of fat in guinea pig livers. Diets 6 and 7, which were the most effective of this series in producing fatty livers in rats, also resulted in a slight elevation of the fat content of the guinea pig livers. However, this was not diminished by the inclusion of choline or methionine in these diets and, consequently, this effect cannot be ascribed to choline deficiency.

The data presented herein should not be interpreted as indicating that the metabolism of phospholipids in the guinea pig is radically different from events in the rat. The possibility exists that the conditions of these experiments permitted sufficient intra-intestinal synthesis of labile methyl compounds to meet the animals' requirements. However, the failure of glycocyamine, like that of nicotinamide,[17] to increase the demand for a supply of methyl groups sufficiently to result in fatty liver formation is surprising. The present findings are compatible with the lack of hepatic choline oxidase activity in this species and suggest that the lack of this enzyme results in a choline turnover so slow as to permit a nutritionally adequate supply of choline from such choline and methionine as was available in the diets of this study. While it may yet be found that the position of choline in the nutrition of the guinea pig is much like that in the rat, the daily requirement of the guinea pig for choline (or its equivalent in methyl groups) is certainly of a lower order of magnitude than that of the rat.

Summary. Seven different choline deficient diets were fed to young rats and guinea pigs. While each of these diets resulted in fatty liver formation in the rats, in no instance was there observed an appreciable accumulation of fat in the guinea pig livers. This fact has been correlated with the lack of hepatic choline oxidase activity in the guinea pig as compared to all species which have, to date, been found susceptible to dietary choline deficiency.

The author's thanks are due to the Duke University Research Council and to The Nutrition Foundation for their support of this work; to Merck and Co., Rahway, N. J., for the crystalline vitamins employed; and to the Wilson Laboratories, Chicago, Ill., for a generous supply of liver fraction L.

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15 Handler, P., *J. Biol. Chem.*, 1946, **162**, 77.

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16829

Glutamic Acid and Vomiting in Dogs: Its Administration into the Portal System and Extremity Veins.*

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The intravenous administration of solutions containing glutamic acid has been shown to produce vomiting both in dogs[1] and in man.[2] Price, Waelsch and Putnam,[3] on the other hand, have given as much as 20 g of glutamic acid orally to epileptic patients per day in divided doses and did not record any incidence of vomiting. The increased tolerance to orally administered glutamic acid may be due either to slow absorption from the digestive tract, or to the fact that products which are absorbed from the gastro-intestinal tract by way of the blood are first conveyed to the liver. In this organ many reactions occur which could destroy free glutamic acid, such as deamination, transamination, or peptide formation. In order to study the detoxifying action of the liver on glutamic acid two methods of administration were used whereby the infusions would enter the portal system before reaching the general circulation. This was accomplished by infusing the solutions containing glutamic acid either directly into the spleen or the portal vein. Using these procedures an attempt was made to determine whether the animals could tolerate glutamic acid better by the intraportal than by the peripheral intravenous route. Studies were also made in an attempt to relate the amino acid nitrogen and urea nitrogen content of the blood with the route of administration, and the production of vomiting.

Methods. Six adult male mongrel dogs were used in this study. Under intravenous nembutal anesthesia the abdomen was entered through a left rectus incision. After freeing the integument over the left half of the abdomen the left external and internal oblique muscles were resected. A rent was made in the transverse muscle and peritoneum through which the spleen was delivered. The hilus of the spleen was sutured to the margin of the peritoneum to prevent herniation of an abdominal viscus through it. The purpose of removing the external and internal oblique muscles was twofold: first, to allow more space for the implanted spleen; secondly, to prevent obstruction of the splenic circulation, either by scar tissue or by spasm of these muscles during an intrasplenic infusion. The abdomen was closed with interrupted cotton sutures.

In 3 animals, at the same time the spleen was transplanted, a small plastic tube† was sutured on the portal vein and brought to the outside through a small incision. The free end of the plastic tube was covered with sterile gauze and taped to the animal's side. This tube served the same purpose as the metal catheters used by London[4] in his angiostomy technic. Thus, by running a needle down this tube it was possible to infuse the glutamic acid solution into the portal vein in the unanesthetized animal without difficulty.

The glutamic acid solution used for all the infusions was prepared by adding 7 g of l +

* A preliminary report of this work was given before the American Federation for Clinical Research, Chicago, October 30, 1947.

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2 Smyth, C. J., Levey, S., and Lasichak, A. G., *Am. J. Med. Sci.*, 1947, **214**, 281.

3 Price, J. C., Waelsch, H., and Putnam, T. J., *J. Am. Med. Assn.*, 1943, **122**, 1153.

† The plastic tubing used was that supplied by the Baxter Company for their disposable infusion sets.

4 London, E. S., Harvey Lectures, 1927-28, p. 208, Williams & Wilkins Co.

TABLE I.
Effect of Route of Administration of Glutamic Acid Solutions on the Production of Vomiting in Dogs.

Dog No.	Wt, kg	Glutamic acid admin. Route	Vol. received, ml	Rate, ml/min	Reaction
1	11.8	I.V.*	150	10.0	Vomited
		I.S.†	200	8.0	0
		I.V.	145	7.2	Vomited
		I.S.	275	11.2	0
		I.V.	145	8.0	Vomited
		I.S.	285	9.5	0
		I.V.	150	8.8	Vomited
2	16.6	I.V.	80	7.5	Vomited
		I.S.	125	8.9	”
		I.V.	58	8.3	”
		I.S.	150	12.5	0
		I.S.	160	10.0	Vomited
		Intestinal vein	135	10.3	Vomited
3	15.4	I.V.	100	11.0	”
		I.S.	175	8.0	”
		I.V.	150	10.8	”
4	12.2	I.V.	150	10.0	”
		I.S.	315	10.5	”
		I.V.	110	9.0	”
		P.‡	200	10.1	”
5	11.8	I.V.	95	9.5	”
		I.S.	155	10.3	”
		I.V.	100	12.2	”
		I.V.	100	9.5	”
		I.S.	160	8.0	”
		P.	300	8.4	” after infusion stopped
6	11.4	I.V.	95	5.0	Vomited
		I.S.	175	5.0	”
		P.	165	4.5	”

* Intravenous.
† Intrasplenic.
‡ Injection into the portal vein by the angiostomy technic.

glutamic acid (Merck)‡ and 2 g of sodium bicarbonate to 500 ml of pyrogen-free saline. The solutions were sterilized by autoclaving.

Approximately 3 weeks after the transplantation of the spleen, the animals received the first intravenous infusion of glutamic acid. The solution was allowed to run into a leg vein at a uniform rate until the animal vomited. At this point the volume infused and the rate of infusion were recorded. Several days subsequent to the intravenous infusion the animal received an intrasplenic injection of glutamic acid. Since the transplanted spleen permitted the palpation of the gland, the needle could be easily directed into this organ. In addition, the intrasplenic position of the needle was confirmed by the aspiration of blood fluid. The solution was allowed to flow into the substance of the spleen at a uniform rate until the animal vomited. In some cases when approximately two times the intravenous dose was given into the spleen and the animal did not vomit, the intrasplenic infusion was discontinued because of danger of overhydration. After a few days

‡ We wish to thank Merck and Co., Inc., Rahway, N. J., for supplying the glutamic acid used in this study.

3 of the animals received a third infusion directly into the portal vein by the angiostomy technic, and the volume of fluid necessary to make the animal vomit was recorded. Some of the animals received more than one test involving a single method of infusion.

Results and discussion. A comparison of the tolerance of animals to glutamic acid administered either intravenously or directly into the portal system is presented in Table I. More glutamic acid could be administered into the portal system without the animals vomiting than could be given into the peripheral venous circulation. In a single case (Dog 2) the glutamic acid solution was injected into an intestinal vein isolated under local anesthesia and a greater tolerance was found for the amino acid when given in this manner than when administered into one of the leg veins.

Since there was no constant difference in either the blood amino acid nitrogen or the urea plus ammonia nitrogen dependent on the route of administration these data will not be presented. Also there was no level of blood amino acid nitrogen following the glutamic acid infusion which was uniformly associated with vomiting.

This work was initiated on the assumption that free glutamic acid could be partially destroyed or removed by passage through the liver. Thus if an infusion of this amino acid were permitted to pass first into the liver it should be better tolerated than if it were given into a peripheral vein. The present study supports this view. The vomiting which followed the intraportal infusions may signify that the capacity of the liver to remove this substance is exceeded. Friedberg and Greenberg[5] have reported the partition of amino acid nitrogen among the various tissues of the rat 15 minutes after the intravenous administration of glutamic acid. They found that the amino acid is slowly cleared from the plasma and at the same time the concentration of the amino acid nitrogen in the liver approached a control value. This could be interpreted as showing that free glutamic acid is rapidly being destroyed or conjugated in the liver.

Summary and conclusions. A method is presented by which the spleen may be readily transplanted subcutaneously in dogs and used for intraportal infusions.

Dogs could tolerate more glutamic acid solution without vomiting when it was given either intrasplenically or directly into the portal vein by using the angiostomy technic, as compared to the peripheral venous administration.

The increased tolerance to glutamic acid solution when given intraportally is attributed to the direct passage of these amino acids to the liver where they may be removed from the circulation.

[5] Friedberg, F., and Greenberg, D. M., *J. Biol. Chem.*, 1947, **168**, 411.

16830

Effect of Atmospheric Carbon Dioxide on Adrenal Cortical Hyperplasia and Associated Changes Due to Stress.

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By submitting animals to anoxia, under a high carbon dioxide partial pressure, Langley,[1] Hailman,[2] and their co-workers succeeded in preventing the activation of the adrenal

* This work was done during the tenure of a Life Insurance Medical Research Fellowship.

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[2] Hailman, H. F., *Endocrinology*, 1944, **34**, 187.

TABLE I.
Effect on Organ Weights* of Exposure to Stress Under a 15% Partial Pressure of Carbon Dioxide.

Group	Treatment	Adrenals (mg)	Spleen (mg)	Thymus (mg)	Lymph nodes (mg)
I	None	9.3 ± 1.1	731.6 ± 41.9	167.6 ± 14	9.1 ± 1.1
II	Spinal cord transection + 15% CO_2	17.9 ± 2.5	472.9 ± 72.9	160.4 ± 9.6	5.7 ± 0.7
III	Immobilization + 15% CO_2	13.9 ± 1.7	274.6 ± 48.4	136.1 ± 8.9	6.4 ± 1.6
IV	Spinal cord transection + normal CO_2	11.6 ± 1.0	539.1 ± 102.8	127.5 ± 16.1	8.9 ± 1.6
V	Immobilization + normal CO_2	15.4 ± 2.9	414.0 ± 42.9	95.3 ± 13.8	7.2 ± 0.4
VI	15% CO_2 without other form of stress	16.1 ± 1.1	645.1 ± 250	149.0 ± 32.4	10.1 ± 1.1

* The mean organ weights are expressed per 100 g of final body weight.

cortex previously observed to follow acute or chronic exposure to decreased barometric pressure alone.[3–18] These investigators accordingly suggest that the stimulus responsible for the adrenal response during anoxia, is the alkaline shift of the acid-base balance which results from hyperventilation and the following acapnia.[19] The object of this study was to examine whether this mechanism, *i.e.* alkalosis, represents a necessary factor to the initiation of the alarm-reaction[20] or whether it is to be considered as but one form of non-specific stress.

Experimental. Forty-eight male albino rats of the Wistar strain weighing between 120 and 140 g at the onset of the experiment were subdivided into 6 groups of 8 animals. All experimental groups were fasted for the duration of the exposure. Group I received no treatment and served as a normal control group. Groups II and III were submitted respectively, under a 15% partial pressure of carbon dioxide, to 2 different forms of stress: spinal cord transection for Group II and immobilization on a board for Group III, both usually giving a well marked response within 24 hours.[21–23] Groups IV and V, which served as experimental controls, were exposed to the same types of stress under normal atmospheric conditions, while the animals of Group VI were subjected, without any additional alarming agent, to the elevated (15%) carbon dioxide tension.

A well ventilated decompression chamber

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23 Selye, Hans, *Endocrinology*, 1937, **21**, 169.

TABLE II.
Probability That the Difference Between Mean Organ Weights Shown in Table I Might Have Occurred by Chance (P).*

Group	II-I	II-IV	III-I	III-V	IV-I	V-I	VI-I
Adrenals	<.01	<.05	<.02	>.20†	>.10†	<.05	<.01
Spleen	<.01	>.20†	<.01	>.20†	>.10†	<.01	<.01
Thymus	>.20†	>.10†	<.01	<.05	<.05	<.05	<.03
Lymph Nodes	<.01	>.10†	<.02	<.05	<.02	>.10†	>.20†

* From Fisher's table of T values.
† Difference not statistically significant.

of the R.C.A.F. standard model was used for maintaining a constant composition of the atmosphere, namely, CO_2, 15%; O_2, 19%; N_2, 66% at a pressure of 760 mm HG. and under normal hygrometric conditions. Samples of the gaseous mixture were analyzed at intervals by the Haldane method. The animals were killed after 38 hours by exposure and the adrenals, spleen, thymus and pelvic lymph nodes were weighed in the fresh state and histologically examined.

Results. The results are summarized in Table I. The probability of a chance occurrence of the differences in the means of the experimental groups (II, III and VI) from those of the normal control group (I) and the experimental control groups (IV and V), is indicated in Table II, in which we have taken as significant those differences for which the probability of occurrence by chance is not greater than 0.05. An increase in the weight of the adrenal glands is evident in both experimental and experimental control groups. In all but Group IV, this increase represents a significant deviation from the untreated control group. Conversely, the spleen, thymus and pelvic lymph nodes show in most groups, a significant decrease in weight which roughly parallels the increase in adrenal weight. The organ weights of Groups II and III, which were exposed to stress under a 15% pressure of carbon dioxide, are not significantly different from those of Groups IV and V which were submitted to the same damaging agents under normal atmospheric conditions. The adrenal hypertrophy and the splenic and thymic involution of Group VI which was exposed to 15% carbon dioxide without additional damaging agent, are significant. The histological examination of the organs revealed in all but Group I various degrees of lipid depletion in the cortex of the adrenals and of nuclear pyknosis in the lymphatic organs. These changes have been repeatedly described in connection with exposure to any non-specific damaging agent.[24]

Conclusion. Our results disprove the possibility that an alkaline shift of the acid-base balance is the necessary prerequisite for the development of the alarm-reaction, as the response of the organs (adrenals, spleen, thymus and lymph nodes) was of the same order in animals submitted to high and normal carbon dioxide tensions. The adrenal cortical hyperplasia as well as lymphatic tissue involution of the animals exposed, without other change in their environment, to a high carbon dioxide atmospheric tension, imply that this factor is in itself an alarming stimulus, perhaps through its action on the acid-base balance of the blood.[25]

This work was subsidized by a grant from the Commonwealth Fund of New York. The author is also indebted to Drs. L. Brouha, L. P. Dugal and A. Desmarais of the Institut d'Hygiène et de Biologie humaine, Université Laval, for the use of their decompression chamber.

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16831

The Role of Copper in Mammalian Pigmentation.

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Copper is an essential dietary factor for maintaining the color of fur in several species of mammals. Keil and Nelson[1] observed that black and piebald rats turned grey when fed on a diet deficient in copper. The original hair color could be restored within a few weeks by daily addition of 50 μg of copper to the diet. These observations were confirmed and extended to other species of mammals.[2-4] The depigmentation of copper-deficient rats and rabbits could not be cured by supplementing the diet with manganese, iron[4,5] or with vitamin B factors.[6] Copper was assumed to act as a catalyst of mammalian pigment formation[7] but there was no direct evidence to support this theory.

Experimental. The experiments which follow were carried out to elucidate the possible role of copper in the production of melanin. One set dealt with the effect of heavy metal catalysts and of inhibitors on melanin formation *in vitro*. In another series copper determinations were carried out on pigmented biological material.

The darkening of buffered solutions of dopa (1 - dihydroxyphenylalanine, Hoffmann La Roche) was measured colorimetrically. All vessels and test tubes were washed with a 1:1 mixture of concentrated HCl and HNO_3 and rinsed 5 times with both distilled and double-distilled water. To avoid introducing traces of heavy metals, barbiturate buffer of pH 7.4 was used throughout and all solutions were made with double-distilled water. Each test tube contained 1 ml of 5:10,000 dopa solution, 1 ml heavy metal salt solution in a 10^{-4} to 10^{-6} M final concentration and 3 ml of the buffer. The test tubes were kept in the incubator at 37° and readings were made in the Klett-Summerson photoelectric colorimeter with filter KS-42 at hourly intervals. Cysteine hydrochloride (2×10^{-3} to 10^{-4} M), phenylthiourea (10^{-4} M) and aqueous extracts of isolated human epidermis[8] were used as inhibitors.

Copper was determined in hair and tissues. The hair was clipped from the animals, washed 5 times with chloroform for several days and then with acetone, dried, weighed and ashed at 500°C. The tissues were defatted according to Greenstein[9] and ashed. The ash was heated with 1 drop of concentrated HNO_3, dissolved in 6 N HCl and the copper determined by the diethyldithiocarbamate method.[9] Iron was estimated by the 2-2′ bipyridine method[10] and manganese by the catalytic method of Kun.[11] All determinations were carried out in duplicate and only data agreeing within 10% were accepted.

The melanin used for copper analysis was prepared from Harding-Passey melanomas of mice by digesting the ground tumor tissue

* American Cancer Society Fellow, 1947-48.

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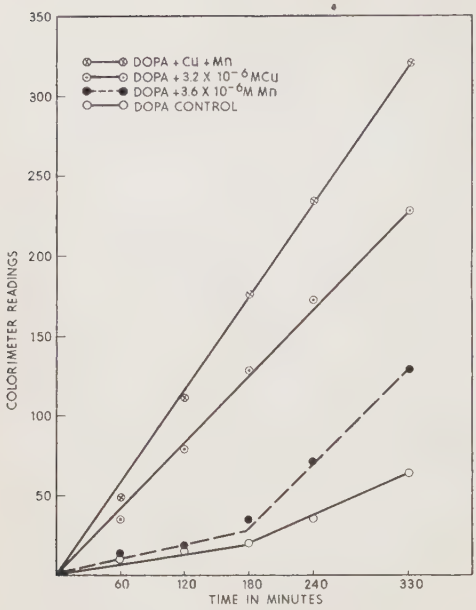

FIG. 1.

Catalytic effect of cupric and manganous ions on the autoxidation of dopa.

with pepsin-hydrochloric acid or with trypsin[12] for 4 weeks. The solution was changed at the end of 2 weeks. The digested tumor tissue was centrifuged and the supernatant fluid was dialyzed against double-distilled water for 7-10 days in the ice-box. The contents of the dialyzing bag were centrifuged, and the black precipitate was washed several times with double-distilled water and analyzed. In another series of experiments ground tumor tissue was kept overnight in 1% NaOH (5 ml/g wet tissue) at room temperature, then boiled for 30 minutes. The dissolved tissue was purified by dialysis, then dried for analysis.

Human epidermis from autopsy material was isolated by the heat method of Baumberger *et al.*,[13] or by treating the skin with 2 N KI solution.[14] Copper determinations were carried out on the defatted dried material.

Results. Catalytic activity of heavy metals on the autoxidation of dopa in vitro. The order of caralytic effect of the metals studied on the autoxidation of dopa was Cu>Co>Ni>Mn>Pb>Fe. Zn had very little effect, Mg and Hg none. The effect is additive (Fig. 1). In agreement with Bernheim[15] it was found that phenylthiourea which inhibits the tyrosine tyrosinase reaction *in vitro*[16,17] and pigmentation *in vivo*[18] had no effect on the autoxidation of dopa. In relatively high concentrations (10^{-4} M) it had only a slight inhibitory influence on the catalytic activity of cupric ion in the oxidation of dopa.

One mole of cupric ion was able to counteract the inhibition of the autoxidation of dopa produced by approximately 500 moles of cysteine (Fig. 2). Experiments with aqueous extracts of isolated epidermis confirmed the findings of Rothman and coworkers of an inhibitory factor in these extracts.[8] The factor was found to be heat stable and dialyzable and antagonized by cupric ion and by p-chloromercuribenzoic acid. This supports the previously advanced theory that the inhibition is due to sulfhydryl compounds.

Copper and iron determinations in hair. As suitable pigmented biological material, the hair of mottled rabbits, guinea pigs and rats

TABLE I.

Copper in μg/g in the Hair of Pigmented and White Areas of the Same Animal.

Animals	Copper content in μg/g hair	
8 rabbits	Grey or black: 35.4 (23.2-41.4)	White: 25.1 (9.3-35.8)
2 "	Light brown: 22.5 (22.2-22.8)	White: 20.6 (20.2-20.9)
5 guinea pigs	Black: 26.2 (9.0-46.0)	White: 15.5 (1.0-47.1)
2 " "	Black: 16.4 (13.0-19.8)	Red-brown: 8.7 (6.6-10.8)
3 " "	Red-brown: 26.5 (18.5-35.5)	White: 26.6 (18.3-36.3)
4 rats	Black: 29.1 (13.3-38.9)	White: 23.5 (13.9-31.6)

[12] Greenstein, J. P., Turner, F. C., and Jenrette, W. V., *J. Nat. Cancer Inst.*, 1940, **1**, 377.

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TABLE II.
Copper in Human Epidermis in μg/g Dry Fat-free Tissue After Separation of Epidermis from Corium.

White epidermis		Corium	Negro epidermis		Corium
No. 1	73.5	35.4	No. 1	50.2	15.2
No. 2	53.0	39.6	No. 2	53.5	24.4
No. 3	88.9	28.7	No. 3	51.9	11.1
No. 4	29.7	12.0	No. 4	49.4	6.2
No. 5	56.3	18.1			
No. 6	142.7	33.0			
No. 7	186.6	31.5			
No. 8	180.2	-			
No. 9	139.4	—			

TABLE III.
Copper Determinations in Harding-Passey Melanomas and in Melanin Prepared from the Tumor.

No.	Wet wt of tumor. g	Water content, %	Cu in μg/g dry fat-free tumor tissue	Cu in μg/g melanin
1	7.10	79.7	11.5	—
2	6.75	79.4	13.5	—
3	5.26	79.0	12.5	50.4
4	5.20	—	26.2	337.9
5	4.60	79.7	15.1	—
6	4.28	78.8	17.5	163.0
7	2.45	81.3	22.4	—
8	1.95	78.5	26.5	—
9	0.52	—	59.2	294.0

was chosen for copper determinations. By comparing white and colored hair from the same animal, individual variations in the copper content due to age, strain, and diet of the animals[19] were excluded. The results are presented in Table I.

The black or grey hair of 6 of the 8 rabbits, 6 of the 7 guinea pigs, and 2 of the 4 rats tested contained significantly more copper than the white or red brown hair of the same animals. No difference was found in the copper content of white and brown hair.

Determinations carried out on guinea pig hair showed that the iron content of the red-brown hair was considerably higher than that of the white hair of the same animal. The mean values in 6 animals were 35.4 μg/g (25.0–46.1) for red-brown hair and 15.4 μg/g (4.5–23.2) for white.

Copper determinations in isolated human epidermis. Isolated human epidermis obtained from autopsy material showed the same large individual variations in copper content as had the hair of animals. Preliminary experiments with Negro epidermis indicated a copper content in the same range as that found in white specimens, but not enough experimental material was available to allow making definite conclusions. The copper content of the epidermis invariably was higher than that of the corium. (Table II).

Copper determinations in Harding-Passey mouse melanomas. The tumor tested has been carried in this laboratory for one year in mice of the dba strain. Copper determinations were made on such tumors and on the kidneys and spleens in normal and tumor bearing animals. In the same animal the copper content of melanomas was below that of the kidneys (71.3 μg/g dry weight) and spleens (52 μg/g). Larger and older tumors had a lower copper content than recently implanted ones and all tumors had a relatively high water content.

The melanin pigment prepared from the melanomas by boiling with NaOH, contained 4 to 13 times more copper than the tumor. (Table III) In the amounts used in the preparation, the NaOH could have accounted for only about 5% of the copper found in the

[19] Cunningham, I. J., *Biochem. J.*, 1931, **25**, 1267.

melanin, a percentage within the experimental error of the copper determination. On the other hand, the melanin pigment prepared by peptic and tryptic digestion yielded a copper content 20 to 95 times higher than the tumor tissue. The difference may have been due to more complete hydrolysis of the tumor tissue, but since both the pepsin and trypsin used had relatively high copper contents, the possibility could not be excluded that some copper in the pigment prepared by digestion was of extraneous origin. These data are therefore not presented. On the other hand, the manganese content of the tumor (57.3 μg/100 g dry fat free tissue) was practically identical with that of the pigment (63.2 μg/100 g) derived from it. Values obtained with liver and kidney were in the same range.

Discussion. The catalytic influence of cupric ions on the autoxidation of dopa has been described by several authors.[7,19] No comparative experiments with other heavy metallic salts have been reported. Sarata[7] observed that copper sulfate exerted a catalytic effect only in a certain range of concentration. In this laboratory the apparent inhibition by relatively high concentrations of copper sulfate, described by Sarata, was found to be due to the use in his experiments of unbuffered solutions; in relatively large amounts, copper sulfate lowered the pH below the neutral range. In some of our experiments, involving the use of buffered solutions, copper sulfate in concentrations which Sarata had found to be inhibitory (5 μg/ml) showed a more powerful catalytic action than in lower concentrations. Copper determinations on the hair of mammals have been made by several authors. In two papers its concentration was reported to be higher in black hair than in white hair from the same animal,[7,19] while in others no such difference was found.[20,21] However, the determinations were on a very limited number of animals. Melanogenesis takes place in the germinative epithelial layer of the hair and even if copper is involved, it may not ascend with the pigment in the growing hair. The higher iron values in red-brown guinea pig hair than in white hair are of interest in view of the fact that an iron-containing red pigment has been isolated from human red hair.[22] The literature has no data on the copper content of isolated human epidermis. Relative values for whole skin, expressed in relative intensities of spectral lines, showed the same large individual variations as observed in our experiments.[23] In bovine epidermis, Cunningham[19] found much larger values than in the dermis of the same animal. He suggested that the skin was an important organ for copper excretion. Since the pigment constitutes such a small fraction of the entire epidermis, a difference in the copper content of pigmented and non-pigmented skin may not be detectable. This would explain the relatively low copper values in the epidermis of the Negro. The low copper values of melanotic tumors agree with the data of other authors.[19,24,25] However, the finding

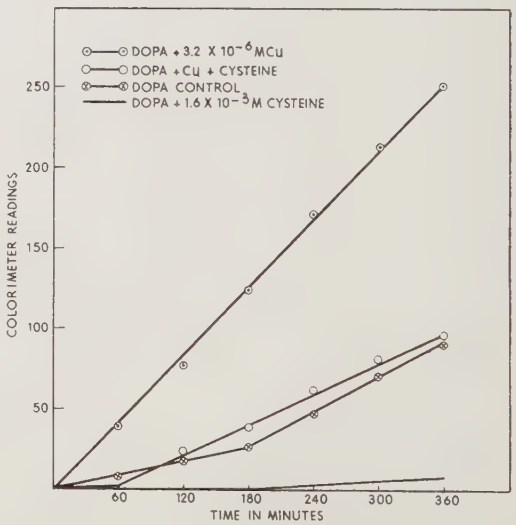

FIG. 2.

Suppression, by cupric ion, of the inhibitory effect of cysteine hydrochloride on the autoxidation of dopa.

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that the isolated pigmented part of the tumor is strikingly high in copper concentration strongly favors the theory that copper acts as a local catalyst in pigment formation, probably as part of an oxidative enzyme, possibly tyrosinase which has recently been demonstrated in melanotic tumors of mice.[26,27] Mammalian tyrosinase contains significant amounts of copper and can be inhibited by substances which combine with this metal.[28] Copper apparently also forms a metallo-organic complex with dopa[29] and may remain attached to the melanin molecule. In this connection it is of interest to note that the ash of octopus ink contains as much as 2% copper.[30]

We advance the theory that copper may promote pigmentation, not only by direct action on the substrate, but also indirectly by oxidizing sulfhydryl groups which inhibit pigmentation *in vitro*,[31] and probably also *in vivo*.[8,32,33] Greenstein *et al.* have indicated that melanin in mouse melanomas is attached to the rest of the pseudoglobulin molecule in the vicinity of sulfur containing amino acids.[12] Our finding that copper, too, is closely linked to the pigment suggests an interaction between copper and sulfhydryl compounds.

Summary 1. Autoxidation of dopa *in vitro* was more strongly catalyzed by cupric ions than by any of the other heavy metallic salts investigated. The order of catalytic activity was Cu>Co>Ni>Mn>Pb>Fe.

2. Black and grey hair of rabbits, guinea pigs and rats generally, but not always, contained more copper than white hair of the same individual. In guinea pigs, red-brown hair contained more iron than white hair.

3. Analysis of the separated layers of human skin showed a considerably higher copper content in the epidermis than in the corium.

4. Melanin from mouse melanomas had a copper content 4 to 13 times higher than the tumor from which it was prepared.

5. The theory is advanced that copper plays a role as a local catalyst in mammalian pigmentation.

The author wishes to thank Dr. Stephen Rothman for his interest and assistance in the present work.

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16832 P

An Antidiuretic Substance in the Blood of Normal and Adrenalectomized Rats.*

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In adrenal insufficiency the diuretic response to water is greatly reduced or absent (reviewed by[1]). An attempt was made to see if this was due to the accumulation of anti-

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

[1] Gaunt, R., *J. Clinical Endocrinology*, 1946, **6**, 595.

TABLE I.
Showing Mean Effect on Water Diuresis Produced by Various Types of Blood Serum.

Series	Type of serum tested: (1 cc sample)	No. animals	% water excreted at indicated min.			Urine chlorides	
			30	60	90 ± S.E.*	mg/cc as NaCl	Total 3 hr
1	Normal—fresh	17	4	15	35 ± 3.56	1.06	11.15
2	Adx. 3-7 days—fresh	26	2	10	26 ± 2.22	1.14	11.82
3	Adx. 9-12 " "	10	1	6	20 ± 4.03	1.29	13.23
4	Normal—drawn 12 hr	16	20	45	66 ± 4.97	0.45	5.56
5	Adx.— " 12 "	5	9	32	51	0.55	7.07
6	Normal saline (no serum)	12	19	45	64 ± 3.67	0.43	6.21

* S.E. $= \pm \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

diuretic substances (called ADS) in body fluids. The use of a modified Heller and Urban[2] technic revealed that the serum of normal animals contained a consistently detectable amount of labile ADS and that its concentration increased after adrenalectomy.

Methods. In testing samples of blood serum for their antidiuretic content, male rats weighing approximately 200 g were fasted but allowed water for 18 hours. Two doses of water, 3 cc per 100 sq cm of body surface, warmed to body temperature, were then administered at hourly intervals to animals in individual metabolism cages. For a 200 g rat each dose was 9 cc. One hour after the administration of the second dose of water the urine was measured. Any rat which, during the 2 hour hydration period, had a urine excretion rate 50% more or less than the mean of the groups of 6 was discarded. This necessitated the elimination of 6%. The rats with a normal diuretic response were then placed over clean funnels, the test material was injected intraperitoneally, and a third dose of water was administered. Following the third dose of water the urine output was measured at 30 minute intervals for 3 hours. The water excreted was calculated as the per cent of the total water given (3 doses) minus that excreted prior to injection of test material. It was found that this method, designed not so much to equalize hydration, as to establish a high diuretic rate, permitted better determinations of ADS than the ordinary Burn assay procedure. The most consistent differences between groups occurred at 90 minutes and figures beyond that time are not tabulated here.

To obtain serum, blood was taken directly from the heart without anticoagulant after induction of ether anesthesia. The blood was quickly centrifuged, serum decanted, and immediately injected into test animals unless otherwise specified.

Results. As seen in Table I, Ser. 1, the injection of 1 cc of serum from normal animals depressed urine volume and increased chloride excretion. If the decanted serum samples were allowed to stand at 5 - 9°C for 12 hours the antidiuretic and chloruretic activity was lost (Ser. 4). The serum of animals adrenalectomized from 3 to 7 days had higher antidiuretic and somewhat higher chloruretic activity than that of normal animals (Ser. 2). Again most of the activity was lost in aged refrigerated serum (Ser. 5). Greater amounts of ADS appeared in animals adrenalectomized for 9 - 12 days (Ser. 3). For the first 18 hours after adrenalectomy rats show a normal diuretic response to small (5 cc per 100 g) water loads but not to larger ones.[1] It was found (Table II) that 5 milliunits of Pitressin† was more effective at 18 hours after adrenalectomy than in dummy-operated controls.

Discussion. While antidiuretic substances have been detected in body fluids in various conditions, results have been in general either

[2] Heller, H., and Urban, F. F., *J. Physiol.*, 1935, **85**, 502.

† Supplied by Dr. D. A. McGinty, Parke, Davis and Company.

TABLE II.
Water Excretion in Rats Receiving Water (5 cc/100 g) and 5 Milliunits Pitressin.

	No. animals	% excreted at following min. 60	120	180 ± S.E.
Dummy-operated	12	5.5	47.5	79.9 ± 3.52
Adx. 18 hr	12	4.1	27.6	55.0 ± 4.50

scattered, inconsistent or controversial.[3-6] The test used here apparently reveals the presence of ADS consistently in normal rat serum. This method has been used by Dr. C. W. Lloyd of Syracuse University Medical College and yielded similar results (unpublished) both in normal human subjects and Addisonian patients. While our data is consistent with the hypothesis that the circulating ADS is of posterior pituitary origin, critical evidence on its site of origin and precise quantitative information regarding its activity are being sought.

Although it is obvious that the greater accumulation of ADS may account in part for the failure of water diuresis in adrenalectomized animals, a factor of equal importance may be the increased sensitivity to—rather than the increased amounts of—ADS. This is indicated by the observed augmented sensitivity to Pitressin after adrenalectomy. Thus, normal water excretion may be a consequence of the balanced interaction of diuretic cortical hormones and ADS of pituitary or other origin (concept of Corey and Britton[7]). After adrenalectomy, the ADS, present in increased amounts, could act unchecked by its normal antagonist. Such interpretations are consistent with recent studies on renal function in salt-treated adrenalectomized rats, which indicate that the failure of water excretion is due primarily to an accelerated tubular reabsorption (Lotspeich[8]). Although a decreased glomerular filtration may be observed in untreated adrenalectomized rats, it will not account for the failure of water excretion at moderately high water loads.[9] If the main factor involved is an active ADS, possibly of posterior pituitary origin, its expected action would be on renal tubular reabsorption. The problem, however, is a complex one because extra-renal factors have also been clearly demonstrated.[1,10]

Summary. The blood serum of normal rats contains a labile antidiuretic and chloruretic substance(s?) which increases in amount after adrenalectomy.

Addendum. Since the above was written additional work, in collaboration with Drs. W. R. Boss and C. M. Osborn, has established that: (1) the ADS in fresh normal rat serum does not affect glomerular filtration, as measured by creatinine clearance, and presumably therefore acts by increasing the renal tubular reabsorption of water; and (2) that no ADS is detectable in the blood of hypophysectomized rats.

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8 Lotspeich, W. D., *Fed. Proc.*, 1948, **7**, 74; and additional data by personal communication.

9 Unpublished work of Drs. W. R. Boss and J. H. Birnie of this laboratory.

10 Birnie, J. H., Eversole, W. J., and Gaunt, R., *Endocrinology*, 1948, **42**, 412.

16833

Glucose Tolerance in Decerebrated Rats After Relatively Long Survival.

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It has been amply demonstrated in acute experiments on rabbits and cats that piqûre or decerebration at the pontile level gives rise within less than an hour to hyperglycemia and glycosuria, and that the increase in sugar is usually maintained for 3 or 4 hours, but may last as long as 9 hours. Claude Bernard[1,2] observed that piqûre was most effective when done bilaterally at levels between tthe emergence of the vagus and acoustic nerves. Donhoffer and Macleod[3] demonstrated that hyperglycemia occurred consistently only when decerebration was done at the pontile level, and they were of the opinion that the diabetogenic center probably was situated in the tegmentum pontis. Brooks[4] presented evidence that the center is situated in the floor of the IVth ventricle just caudal to the middle of the brachium pontis and very close to the vasomotor center.

According to Donhoffer and Macleod,[3] midbrain decerebration leads to little or no hyperglycemia, an observation in accord with those of Olmsted and Logan,[5] Bazett, Tychowski and Crowell,[6] Peterson,[7] and Noltie.[8] On the other hand, a high and well sustained hyperglycemic response to midbrain decerebration has been noted by Anderson *et al.*[9] and Evans, Tsai and Young.[10]

The medulla oblongata also has been subjected to piqûre or other lesion in an effort to locate a diabetogenic center. Brugsch, Dresel and Lewey[11] came to the conclusion that the dorsal nucleus of the vagus was the center in point. On the other hand, Hiller[12] and Hiller and Tannenbaum[13] found relatively little increase in blood sugar following damage to this nucleus, and they contended that no blood-sugar-raising (or diabetogenic) center, as such, exists in the central nervous system, but their view depends on what definition the term "center" is given. According to Donhoffer and Macleod[3] and Macleod,[14] only a slight or moderate degree of hyperglycemia ensues after injury to the medulla oblongata.

The rat has been little used in experiments on decerebration-induced or piqûre-induced hyperglycemia. Bell, Horne and Magee[15]

* With the technical assistance of Mrs. Ann Alvey, Mr. Vivian R. Loving, and Mr. William H. Good.

This work was begun in the Department of Physiology at Johns Hopkins University Medical School. The authors wish to thank Dr. Philip Bard and Dr. Reginald B. Bromiley for their invaluable help.

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2 Bernard, C., *Leçons sur la physiologie et la pathologie du système nerveux*, vol. 1, J.-B. Baillière et fils, Paris, 1858, pp. 397-447.

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11 Brugsch, T., Dresel, K., and Lewey, F. H., *Z. f. exp. Path. u. Therap.*, 1920, **21**, 358.

12 Hiller, F., *Münch. med. Wchnschr.*, 1930, **1**, 836.

13 Hiller, F., and Tannenbaum, A., *Arch. Neurol. and Psychiat.*, 1929, **22**, 901.

14 Macleod, J. J. R., *Bull. Johns Hopkins Hosp.*, 1934, **54**, 79.

15 Bell, D. J., Horne, E. A., and Magee, H. E., *J. Physiol.*, 1933, **78**, 196.

TABLE I.
Blood Sugar Values Within Four Hours After Pontile Decerebration.*

Rat No.	Blood sugar (mg %)			
	Preoperative	Postoperative		
	(under anesthesia)	Immediate	1 hr	4 hr
Decerebrated rats				
651	—	—	209	140
650	74	51	208	219
654	84	68	64	123
655	80	155	118	196
656	87	142	218	99
Control: partially decorticated rats				
507	—	—	86	84
300	59	86	86	78
301	77	156	95	58
302	70	155	87	72

* The extent of the lesions was verified on gross examination post mortem.

found that pontile decerebration caused no increase in blood sugar in fasted rats or in those on a well balanced diet, but did occur over a 2-hour period if they had been fed a diet rich in carbohydrates. They were unable to elicit hyperglycemia by midbrain decerebration.

All the decerebration experiments referred to were acute ones, the animals generally surviving not more than a day or two. It is the purpose of this communication to describe the effects of decerebration on the glucose tolerance of rats which survived the operation for from 4 to 20 days.

Methods. Young adult female rats of the Sprague-Dawley strain were used in this study. All had had access to food up to the time of operation. Sodium pentobarbital, 2.5 mg per 100 g body weight intraperitoneally, was the general anesthetic employed. Novocaine (2.0% solution) was used to infiltrate the scalp immediately before opening the skull. Just preceding the operation, 0.05 cc of calcium gluconate per 100 g body weight was given intravenously to facilitate clotting. Decerebration was done at pontile and midbrain levels. Using the dorsal approach, wedges of brain tissue were removed by suction, and an incision then made downward to the base of the skull.

There were two objectives in this study: a) to determine the blood sugar level within a short time after decerebration, and b) to study glucose tolerance after the lapse of several days.

a. In one series of rats, blood sugar levels were determined at the following intervals: 1) preoperatively, just after the animals had been anesthetized, 2) immediately following operation, 3) one hour after operation, and 4) 4 hours after operation.

b. In the other series the animals were given 10 cc of 10% glucose in saline parenterally 6 hours after decerebration. Twenty-four hours postoperatively they were placed on a regimen consisting of 10 cc liquid diet by stomach tube twice daily and 10 cc of 10% glucose in saline and 2500 units penicillin parenterally once a day. From the start they were kept in an incubator at a temperature of 27°C, and under these conditions their rectal temperature was usually 33 to 34°C. Serving as controls were 3 rats, one of which was partially decorticated, one hemidecerebrated, and one normal. These were kept on the same regimen as the experimental animals.

The glucose tolerance test was done on the animals at various times from the 3d to the 23d day after operation. For 17 hours before the test the animals were fasted; they also received no water until one hour before the test, when they were given 10 cc of physiologic solution of sodium chloride by the subcutaneous route. Just before the test was begun, the animals were lightly anesthetized by sodium pentobarbital 2.5 mg per 100 g

TABLE II.
Data on Glucose Tolerance and Autopsy Findings in Decerebrated Female Rats and Controls.

Rat No.	Wt (g)	Operation	Postoperative data: Day	Wt (g)	Glucose tolerance (blood sugar in mg %): Fast.	30 min.	60 min.	90 min.	120 min.	Survival (days)	Died (D) or sacrificed (S)	Autopsy data: Wt (g)	Autopsy data
						Experimental							
343	174	Pontile Decerebration	4	158	120	—	278	398	456	18	D	157	Virtually complete destruction of pons to lower level of trapezoid body.
323	192	Pontile Decerebration	3	205	141	336	442	464	480	4	D	203	Decerebration at midpontile level grossly; microscopic examination not done.
312	187	Pontile Decerebration	7	172	108	117	380	440	504	14	S	169	Virtually complete destruction to lower level of pons.
336	140	Pontile Decerebration	3 6	138 141	84 97	285 247	271 300	— 305	— 326	9	D	126	Complete destruction of pons to level of 7th nerve.
309	216	Midbrain Decerebration	5	196	85	304	420	170	140	20	S	171	Softening of tissue of rostral midbrain equivalent to midbrain decerebration.
						Control							
351	153	Partial Decortication	1 23	143 136	85 >35	111 105	141 140	— 136	— 136	23	S	136	Brain stem intact except superficial softening of dorsal thalamus bilaterally and dorsal epithalamus at one level.
304	169	Pontile hemidecerebration	7 13	134 128	101 79	222 162	204 88	224 80	242 85	13	S	128	Lateral 1/3 to 3/4 of brain stem destroyed unilaterally to lower level of trapezoid body.
318	175	None	5	170	67	193	243	248	229	18	S	165	No histologic changes observed.
43 normal rats (avg)			—	182	76	154	182	179	183	—	—	—	—

body weight. They were then placed in an animal holder with the tail resting on a warm plate. Blood was collected from the tip of the tail. After the fasting blood sample was taken, 5 cc of 20% glucose solution per 100 g body weight were given by stomach tube. Blood samples were taken every 15 minutes for 2 hours. During the glucose tolerance test the animals were quiet, and the rectal temperatures of the decerebrated animals were between 33° and 35°C. Over the period of several weeks during which these tests were carried out, glucose tolerance was done also on 43 normal female rats of the same age. For blood sugar determinations the micro-method of Haslewood and Strookman[16] was used.

At autopsy the brains were fixed in 10% formalin. Blocks were frozen and serially sectioned at 45 microns by the method described by Marshall,[17] and an average of 50 sections per case were stained, one-half by cresyl violet and the other half by a modified Weil myelin sheath method.

Results. a. Blood sugar values within 4 hours after pontile decerebration are indicated in Table I. In neither the decerebrated nor the partially decorticated animals was there an elevation of blood sugar following anesthetization. Immediately after operation, which required about 30 minutes, the blood sugar was somewhat elevated in about half the experimental and control groups. Subsequently there was hyperglycemia in the experimental animals, but not in the controls.

b. Glucose tolerance in rats of longer survival is shown in Table II. The studies were made on these animals from the 3d to the 7th day after operation. The decerebrated rats showed a fasting blood sugar which tended to be normal, but had a decreased tolerance for fed glucose, the blood sugar rising to levels between 450 to 500 mg % at the end of the second hour, except in one animal (rat 336) in which it was 326 mg %. The fifth rat in this series was subjected to decerebration at the midbrain level; it also showed a diminished tolerance for fed glucose, but the highest peak in the blood sugar curve appeared after one hour, and by the second hour the blood sugar level approached normal. The partially decorticated and hemidecerebrated control rats showed glucose tolerance curves which were in the normal range when comparison is made (1) with the values in an unoperated animal (rat 318) subjected to the same regimen as the experimental animals, and (2) with the average values of 43 normal rats which had been fed *ad libitum.*

In order to rule out extraneous factors affecting blood sugar, such as the handling of the decerebrated animal during the glucose tolerance test, a sham test was carried out; it differed from the regular test only in that water rather than glucose solution was given by stomach tube. That the factor of handling was of no import is indicated by the observation that blood sugar values prior to the giving of the water and every 15 minutes thereafter for 2 hours were found to range between 94 and 119 mg%.

Discussion. The experiments herein described differ from those previously reported in that the blood sugar studies were done on the 3d to the 7th day after decerebration. The tests were performed while the animals were in a "healthy state," as indicated by the length of the subsequent survival periods, which in one case was as long as 20 days. It will be necessary to repeat the work on decerebrate animals of longer survival before the duration of the disturbance in carbohydrate metabolism can be determined.

Another aspect of the work was the determination of blood sugar values during the few hours after decerebration. Hyperglycemia of moderate degree over about 4 hours occurred consistently in animals which had had access to food up to the time of operation. This observation differs from that of Bell, Horne and Magee, previously referred to, who found that hyperglycemia occurred only in animals fed a rich carbohydrate diet: ours had been on a standard stock diet. Thus, it would appear that the rat is no different from the rabbit and cat so far as the induction of

[16] Haslewood, G. A. D., and Strookman, T. A., *Biochem. J.*, 1939, **33**, 920.

[17] Marshall, W. H., *Stain Technol.*, 1940, **15**, 133.

hyperglycemia by decerebration is concerned.

This study confirms the well authenticated observation that disordered carbohydrate metabolism is most striking when decerebration is done at the pontile level. Unilateral piqûre of the floor of the IVth ventricle has been reported by several workers, including Claude Bernard, to give rise to transient hyperglycemia, but in one of our control animals (rat 304), in which the pons and midbrain were destroyed unilaterally up to the midline, the glucose tolerance was normal.

Under autopsy data in Table II, only the lower levels of the lesions are indicated, for these are regarded as the significant ones. At operation, considerable brain tissue was removed in order to be sure that decerebration was complete. In each of the animals decerebrated at the pontile level, autopsy studies disclosed either softening or absence of the midbrain, the epithalamus, the superior cerebrum, the hippocampus, and part or all of the thalamus, bilaterally. Free from lesions were the pituitary gland, hypothalamus, subthalamus, and lenticular nuclei, though in one instance (rat 343) the superior part of the hypothalamus at the infundibular level was softened. In the animal listed as having been subjected to midbrain decerebration (rat 309), the original intent was to do a decortication and have the animal serve as a control, but microscopic examination of serial sections of the brain disclosed destruction of the posterior thalamus, the most superior part of the hypothalamus, the pretectal region, the inferior colliculi, and the medial geniculate bodies, with softening of the lateral two-thirds of the rostral midbrain bilaterally.

Summary. Rats decerebrated at the pontile level exhibited a markedly elevated glucose tolerance curve 3 to 7 days after operation. One subjected to midbrain decerebration showed a shallower and less sustained curve. Hyperglycemia occurred in rats during the first few hours after pontile decerebration, which is in confirmation of previously published results on rabbits and cats.

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Effect of an Analogue of DDT on Experimental Murine Typhus.

FLORENCE K. FITZPATRICK. (Introduced by L. Earle Arnow.)

From the Virus Department of the Medical Research Division, Sharp and Dohme, Inc., Glenolden, Pa.

In a search for rickettsiostatic agents that might be more effective than para aminobenzoic acid (PABA), a large group of miscellaneous substances has been tested in mice infected with murine typhus. The results obtained with one of these, 1,1,1-trichloro-2, 2-bis(para-nitrophenyl) ethane, a nitro analogue of DDT, seem to warrant a brief report at this time.* This compound, which will hereafter be referred to as the nitro analogue, was being tested in mice by Kikuth in Germany at the time of the surrender. Nothing is known of his experiments except his notation that the compound was more effective against murine typhus than was methylene blue, and that it was of low toxicity.[1]

We have been successful in treating mice infected with murine typhus with material prepared in this laboratory.† Mice of the

* This substance was named Nitrogesarol by the Germans, a term which would erroneously lead to the belief that it was obtained as the result of the nitration of DDT.

[1] Publication Board Reports, Dept. of Commerce, Report No. 248, p. 63.

† We are indebted to Dr. E. J. Cragoe, Jr., of the Organic Chemistry Department for the preparation of this compound.

TABLE I.
Comparison of Nitro Analogue at 0.5% and PABA at 2.0% Levels.*

	Nitro analogue		PABA		Controls	
			No. of mice dead on day			
Exp. No.	7	12	7	12	7	8
1	0/6 †	2/6	0/6	4/6	3/6	6/6
2	1/14	4/14	3/14	14/14	12/14	14/14
3	1/6	6/6	1/6	4/6	5/6	6/6
4	0/9	3/9	0/9	5/9	7/9	9/9
5	0/6	4/6	0/6	6/6	6/6	—
6	0/6	1/6	0/6	4/6	9/9	—
7	0/6	6/6	0/6	4/6	6/6	—
Totals	2/53	26/53	4/53	41/53	48/56	56/56

* Levels of 0.5 and 2.0% in the diet represent average drug consumptions of 10 and 40 mg, respectively, per mouse per day.

† Numerator denotes number of mice dead; denominator, number of mice used.

dba strain[2] weighing from 11 to 15 g were infected by the intraperitoneal route with 0.25 ml of a 6 to 10% brain suspension that had been prepared from infected brains kept frozen at –60°C. Treated mice received the drug, which was incorporated in ground Rockland chow for 7 days, beginning at the time of infection. Surviving treated mice were observed until death or recovery had occurred. Most untreated controls were dead by the seventh day. Table I summarizes experiments in which the nitro analogue and PABA were compared. It reveals that a high rate of survival during the period of drug ingestion was obtained with both agents, though the level of drug fed in the case of the nitro analogue was only equal to a quarter of the PABA required to achieve the same result. When the drugs were withdrawn on the 7th day there were more delayed deaths in the PABA-treated mice.

In preliminary toxicity tests no untoward symptoms were observed in 20 mice for a week following a single oral dose of 5 g/kg of the compound suspended in tragacanth.

In contrast with the results obtained with its nitro analogue, DDT was found to be highly toxic at levels of 0.4 mg/mouse/day and higher. At a concentration of 0.2 mg/mouse/day, a level tolerated by the mice, no rickettsiacidal activity was observed.

Summary. The nitro analogue of DDT when given in the diet for 7 days to mice infected with murine typhus resulted in a degree of survival on the 7th day equal to that obtained when 4 times the amount of PABA was administered. After the 7th day, when the drug was withdrawn, more PABA-treated mice succumbed, indicating that the nitro analogue is a better rickettsiacidal agent. The oral toxicity of the compound for mice appears to be of a low order.

2 Moragues, V., and Pinkerton, H., *J. Exp. Med.*, 1944, **79**, 35.

16835

Effect of Dibenamine on Renal Function.*

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Clinical interest[1-3] in Dibenamine (N,N-dibenzyl-β-chloroethylamine) has made a full understanding of its toxicity essential. Previous reports from his laboratory[4,5] indicate two major types of toxicity: (1) central nervous system stimulation, particularly when the drug is administered rapidly by the intravenous route, and (2) local tissue damage after subcutaneous or intramuscular administration. Before Dibenamine was made available for clinical trial, a histological study of the organs of rats receiving maximum tolerated or lethal doses for protracted periods was undertaken;[6] this study failed to reveal any renal damage even in animals dying from chronic Dibenamine administration.

The recent report of Ogden[7] that Dibenamine produced renal failure in one dog has questioned the safety of this agent. The present work was undertaken in an effort to evaluate more exactly the effect of Dibenamine on renal function.

Methods. Experiments were carried out on 5 female dogs weighing between 10 and 15 kg which were trained to lie quietly with only loose restraint during the collection periods. Glomerular filtration was determined by creatinine clearance and renal plasma flow by sodium p-aminohippurate (PAH) clearance. Approximately constant plasma levels of creatinine and PAH were maintained by the continuous infusion (about 8 ml/min) of 5% glucose solution containing creatinine and PAH. The plasma creatinine level was maintained at about 10 mg % while the PAH level was usually below 2 mg %. Bladder urine was collected through a rubber mushroom catheter, and at the end of each 10-minute collection period the bladder was washed with distilled water. Blood samples were taken at the midpoint of each urine collection period, by means of a syringe moistened with heparin solution. Creatinine was determined by the alkaline-picrate method[8] and PAH by a modification[9] of the method of Bratton and Marshall.[10]

Dibenamine‡ (20 mg/kg) was administered intravenously once each week for 6 weeks. The animals were divided into two groups. Group A (3 dogs) received Dibenamine in 100 ml of 0.9% NaCl solution over a period of 45 to 60 minutes. Group B (2 dogs) received the entire dose of Dibenamine in one to 5 minutes, after preliminary sedation with 15 mg/kg pentothal sodium intravenously to reduce the severity of the convulsions which

* Aided by a grant from Givaudan-Delawanna, Inc.

† Research Fellow of the Chinese National Institute of Health. Presented in partial fulfillment of requirements for the degree of Master of Science.

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‡ The Dibenamine employed in these experiments was supplied by Givaudan-Delawanna, Inc., as a 5% solution in acidified alcohol-propylene glycol in sterile ampuls prepared for clinical investigation.

TABLE I.
Renal Function in Dogs Before and After Dibenamine Administration.

Dogs		Group A						Group B			
		1 (12.3 kg)		2 (13.4 kg)		3 (14.2 kg)		4 (13.6 kg)		5 (10.0 kg)	
		G.F.R. ml/min.	R.P.F. ml/min.	G.F.R. ml/min.	R.P.F. ml/min.	G.F.R. ml/min.	R.P.F. ml/min.	G.F.R. ml/min.	R.P.F. ml/min.	G.F.R. ml/min.	R.P.F. ml/min.
Control		46.6	130.3	61.7	181.9	56.2	168.6	57.9	163.6	42.2	121.5
,,		45.6	125.3	60.4	178.9	57.0	166.9	57.6	160.3	41.8	122.3
,,		45.9	126.0	60.5	180.5	58.2	172.1	57.7	162.2	41.4	121.9
After Dibenamine											
Successive weekly inj.*	1st	46.1	110.8	58.8	162.6	56.2	165.8	58.2	150.2	42.8	144.0
	2nd	45.0	121.5	64.0	191.8	57.6	172.3	57.5	152.0	44.3	125.5
	3rd	47.6	126.5	61.7	185.2	57.0	178.4	57.2	144.2	41.0	126.4
	4th	47.6	130.0	63.2	170.0	58.8	160.4	57.7	194.5	42.4	127.8
	5th	47.8	130.6	60.1	179.2	58.8	158.2	56.9	217.2	44.4	130.9
	6th	(42.0) †	(86.2)	(62.4)	(144.6)	(56.5)	(124.7)	(57.1)	(162.9)	(44.5)	(118.2)
		47.7	127.7	60.6	188.5	56.9	155.7	57.0	200.8	44.4	132.5

* Renal function tests performed one week after each injection.

† Figures in parentheses determined at height of adrenergic blockade, 1½ hours after 6th Dibenamine injection. Note return to normal one week later.

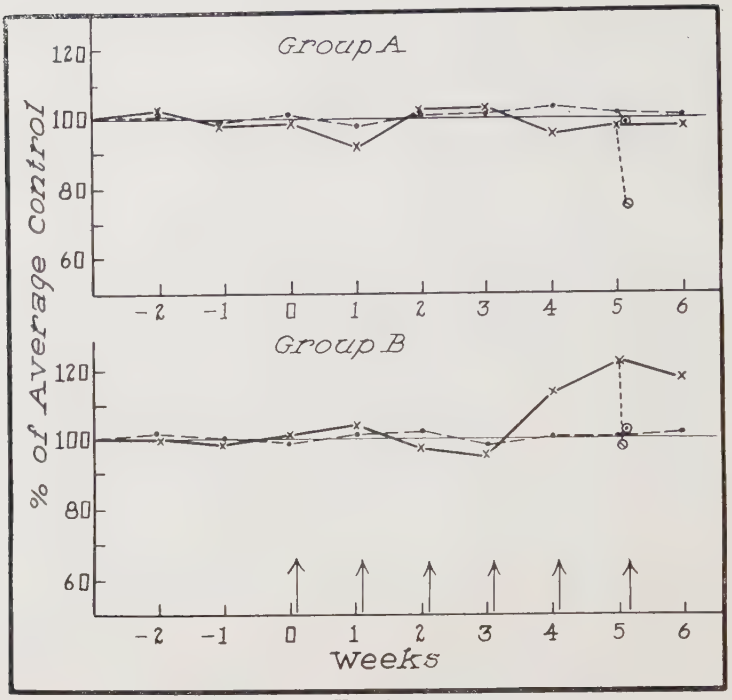

FIG. 1.

Mean glomerular filtration (•——•) and renal plasma flow (x——x) plotted as per cent of average control values for each group of dogs. Dibenamine (20 mg/kg) administered intravenously at each arrow. Circled points indicate determinations made 1½ hours after Dibenamine administration.

result from the rapid administration of such large doses of the drug. The vein was flushed with 0.9% NaCl solution at the end of the injection to prevent thrombophlebitis. Renal function was redetermined one week after *each* Dibenamine injection, and also once in each animal during the height of the Dibenamine action (1½ hours after the 6th weekly injection). Determinations of the mean systemic arterial pressure were made on several animals before and after Dibenamine administration by the use of a capillary mercury manometer and direct femoral artery puncture.

Results. The results of all clearance tests are shown in Table I and Fig. 1. In Fig. 1, mean rates of glomerular filtration and renal plasma flow are plotted as percentage of the average of three control determinations for each animal.

No persistent alterations in glomerular filtration or renal plasma flow occurred in any of the animals, with the exception of a moderate increase in renal plasma flow in one dog receiving rapid injections of Dibenamine. This increased plasma flow was accompanied by a decrease in the filtration fraction. Its significance is difficult to evaluate.

Clearance determinations made during the height of the adrenergic blocking action of Dibenamine showed a consistent reduction in renal plasma flow, which was essentially the same in the two groups; but no reduction in the glomerular filtration rate occurred. In no case did the reduction in renal plasma flow persist until the following test, *i.e.*, after wearing off of the adrenergic blocking action of the Dibenamine.

Determinations of mean systemic arterial pressure indicated about a 30% decrease 2 hours after Dibenamine administration (*e.g.*, 145 to 100 mm Hg).

With the large doses of Dibenamine employed, side-effects were not uncommon. Animals receiving the drug by slow infusion always developed hyperpnea and showed other

evidence of mild central nervous system stimulation. They salivated profusely and usually defecated. Vomiting occurred at least once in each animal, but was never observed later than the third injection in any series. As expected, the immediate toxic reactions were severe when the drug was administered in a period of 5 minutes or less. Vomiting and defecation occurred regularly and clonic convulsive movements developed after each rapid injection in spite of the barbiturate sedation. These animals were prostrated for a period of 24 to 48 hours, but appeared normal before the next weekly test. None of the animals showed any significant weight loss during the course of the experiment.

Discussion. A careful reevaluation of the renal toxicity of Dibenamine was made necessary by the work of Ogden[7] who reported that 2 weekly intravenous administrations of 20 mg/kg Dibenamine caused persistent marked reductions in renal plasma flow and glomerular filtration in one dog. He also described histological evidence of tubular degeneration. A subsequent report by the same author[11] discussed 2 additional animals given more numerous injections of Dibenamine with successively less evidence of renal damage. Ogden reported symptoms of shock in his animals immediately after the Dibenamine injections. Although injection rates were not given, it seemed possible that the alleged renal damage was related to the too rapid injection of the drug, particularly in the first animal studied. The much increased toxicity of Dibenamine after rapid intravenous injection has been repeatedly reported and warned against.[2,4,5]

Because of the possibility that a failure to observe precautions regarding the rate of Dibenamine infusion was responsible for the reported renal damage, dogs in the present experiments were divided into two groups. Group A received the drug by slow infusion with minimal side-effects. Group B received the entire dose of drug within 5 minutes for three injections, and when this procedure failed to produce persistent changes in renal function the remaining injections were completed within 1 minute each. The latter procedure provided the maximum stress possible with this dose of Dibenamine, but it failed to produce detectable renal damage.

These observations on renal function provide confirmation by more sensitive tests of the conclusion from histological observations[6] that even massive doses of Dibenamine do not produce significant renal pathology.

In the absence of a detailed report on the experiments of Ogden, we are unable to offer any explanation for the discrepancy between his results and our own. The fact that he observed decreasing renal damage in successive animals suggests that some factor in the experimental procedure may have been responsible for his findings, but our data indicate that the rate of injection is not a major factor.

The consistent decrease in renal plasma flow but not glomular filtration during the adrenergic blocking action of Dibenamine deserves special mention. This effect was observed almost equally after both slow and rapid injection. The decrease in renal plasma flow can probably be accounted for on the basis of vasodilatation in vascular beds other than renal, and the consequent lowering of the systemic arterial pressure. The decrease in blood pressure observed by direct femoral artery puncture in several animals was greater than that usually observed in anesthetized animals[4,5] or normal recumbent humans.[1,2] It is perhaps correlated with the initially high mean systemic arterial pressure in dogs and suggests that sympathetic vasomotor tone is an important factor in maintaining their blood pressure.

The increased filtration fraction (avg. 21.7%) after Dibenamine indicates a differential effect on afferent and efferent arterioles. Dibenamine has not been shown to have any direct vasconstrictor action, and the similarity of the changes observed with fast and slow injections of the drug suggests that any renal vasodilation was probably due to the adrenergic blockade *per se*. If the Dibenamine induced changes in filtration fraction are explained entirely on the basis of a release of vasomotor tone (adrenergic blockade), it

[11] Ogden, E., Report at Meeting of Fed. of Am. Soc. for Exp. Biol., 1948.

must be assumed that the tonic neurogenic constriction of afferent arterioles is greater than that of efferent arterioles. This interpretation is a variance with the conclusion of Chasis, *et al.*[12] that the efferent arteriolar bed is the major site of vasomotor regulation in the human kidney, and the conclusion of Hiatt[13] that the renal circulation of the dogs has little or no vasomotor tone.

It is clear that our present knowledge of the complex mechanism controlling renal blood flow is inadequate to provide a definitive explanation of the mechanism by which Dibenamine increases the renal filtration fraction. It is possible that in our experiments the lowered blood pressure and renal blood flow after Dibenamine induce a sufficient production of renin to alter the picture of pure vasomotor blockade. Additional experimental work is required to elucidate the mechanism of the Dibenamine induced increase in filtration fraction. Clarification of this problem may also provide a better understanding of renal vasomotor regulation.

Summary. 1. Six weekly intravenous injections of Dibenamine (20 mg/kg) failed to produce any detectable renal damage as measured by creatinine and PAH clearances in 5 trained, unanesthetized female dogs, even when the entire dose was injected within one minute so as to produce maximum toxic side-effects including prostration for 24 to 48 hours.

2. During maximal sympathetic blockade by Dibenamine, renal plasma flow was reduced 10 to 34% (average 21%), probably on the basis of reduced systemic arterial pressure. However, the filtration fraction was sufficiently elevated to provide unaltered glomerular filtration. These alterations never persisted beyond the period of active adrenergic blockade.

3. We have been completely unable to substantiate the observations of Ogden[7] on the renal toxicity of Dibenamine or to find any renal basis for his warning regarding its use. Even large doses of Dibenamine administered very rapidly appear to have no significant reno-toxic action.

12 Chasis, H., Ranges, H. A., Goldring, W., and Smith, H. W., *J. Clin. Invest.*, 1938, **17**, 683.

13 Hiatt, E. P., *Am. J. Physiol.*, 1942, **136**, 38.

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Enhancement of Penetration of Penicillin into Inflamed and Normal Mucous Membrane by Hyaluronidase.

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Duran-Reynals[1] first described the presence in testicular extract of a factor which is capable of facilitating the spread of vaccine virus in the skin of rabbits. Since then, this factor has been shown to be identical with hyaluronidase.[2] The enzyme has been found capable of spreading such diverse substances as vaccine virus, dyes, india ink, rabies virus, hemoglobin, diphtheria toxin, glucose and methemoglobin. It appeared to the authors that it would be of interest to determine whether penetration of penicillin into a mucous membrane might also be enhanced by hyaluronidase particularly since therapeutic failure with penicillin is sometimes ascribed to the inability of the antibiotic to reach deep-seated foci of infection. Our studies were carried out as follows:

1 Duran-Reynals, F., *Compt. Rend. Soc. Biol.*, 1948, **99**, 6.

2 Chain, E., and Duthie, E. S., *Brit. J. Exp. Path.*, 1940, **21**, 324.

TABLE I.

Blood Penicillin Levels in Patients with Chronic Suppurative Sinusitis Following the Instillation of 200,000 Units of Crystalline Penicillin G into the Antrum. With and Without Hyaluronidase. (Units/ml serum.)

Patient	Hyaluronidase	Time after instillation		
		30M	60M	120M
1.	0	1.33	.8	
2.	0	.8	.57	
3.	0	1.33	.8	
4.	0	.8	.44	
5.	0	.133	.08	
6.	0	1.0	.8	
7.	0	.57	.4	
8.	0	.44	.133	
9.	0	1.33	1.0	
10.	0	1.0	.66	
11.	0	.8	.44	
12.	0	.2	.2	
13.	0	1.0	.8	
14.	0	.8	.5	
15.	0	.1	.066	
	H-1	.66	.4	
16.	0	.5	.44	
	H-1	1.33	1.0	
17.	0	.57	.44	
	H-1	1.0	.66	
18.	0	.5	.44	
	H-1	1.0	.88	
19.	0	< .05	< .05	
	H-11	.4	.133	
20.	0	.1	.2	.2
	H-11	.5	.5	.44
21.	0	< .05	< .05	
	H-11	.1	.066	
22.	0	—	.2	< .05
	H-11	.2	.2	.2
23.	0	.8	.57	
	H-11	1.0	.8	
24.	0	.2	.1	
	H-11	.5	.4	
25.	0	1.0	.8	
	H-11	.8	.57	
26.	0	.2	.133	
	H-11	.4	.2	

Experimental. Two groups of patients were selected for this investigation. One group consisted of 26 patients with varying degrees of chronic suppurative disease of the maxillary sinus. The other consisted of 5 subjects clinically free of sinus disease in whom antral lavage yielded a clear return.

After preliminary cocainization followed by suction and cleansing, an antral cannula was inserted into the maxillary sinus through the middle meatus. The sinus was then irrigated with physiological saline solution and the excess fluid was drained off as far as possible by tilting the head so as to make the natural

TABLE II.
Blood Penicillin Levels in Normal Subjects Following the Instillation of 200,000 Units of Crystalline Penicillin G into the Antrum. With and Without Hyaluronidase. (Units/ml serum.)

Patient	Hyaluronidase	Time after instillation 30M	60M	120M
A	0	.4	.08	
B	0	1.3	1.3	
C	0	.44	.2	.05
	H-11	.66	.5	.4
D	0	.5	.4	.057
	H-11	1.0	.8	.57
E	0	.5	.44	.1
	H-11	.8	.66	.3

ostia dependent. Air under 15 lb pressure was blown into the cavity in order to further empty the sinus. 200,000 units of crystalline penicillin G, dissolved in 2-3 cc of saline solution, were instilled into the sinus. Large doses were employed in order to achieve adequate blood levels so that comparisons with and without hyaluronidase would be definitive. With the crystalline preparation unlike the older amorphous penicillin local irritation was absent or minimal. The head was now tilted towards the irrigated side and kept in this position throughout the period of observation in order to prevent escape of the antibiotic. After the cannula was withdrawn a gelfoam pack was inserted into the middle meatus as an additional safeguard against leakage. In the first 3 patients tested the instilled penicillin was colored with gentian violet and upon removal of the white gelfoam pack, the absence of any stain upon it was an indication that leakage had not occurred.

Blood samples at 30, 60 and in some instances 120 minutes after the antral instillation were drawn from the antecubital vein for penicillin assay. The blood penicillin level was considered a good index of penetration since the only way in which the antibiotic could reach the blood stream was by traversing the mucous membrane. The method of assay was a tube dilution method using *Staphylococcus aureus* H as the test organism and fresh meat extract broth as the medium. The minimal concentration of standard penicillin* required to inhibit an inoculum of 5×10^2 *Staphylococcus aureus* H cells was 0.02 units per ml. All titrations of serum levels were compared with this standard.

The effect of hyaluronidase on penicillin penetration was investigated in a number of patients in each group by administering after a few days the same dose of penicillin with the addition of hyaluronidase. Blood samples were again assayed for penicillin and the values found with hyaluronidase and without its use were compared. Two batches of hyaluronidase† were utilized. The first, designated H-1, was employed in a dose of 20 turbidity reducing units. The second, H-11, was given in doses of 42 units since preliminary trials revealed this batch to be not as potent as H-1.

Results. The instillation of large doses of crystalline penicillin into the diseased and normal antrum was well tolerated by all the patients under study. There were no subjective complaints and no evidence of local irritative reaction following treatment. One patient proved to be allergic to penicillin and 24 hours after the instillation exhibited a diffuse skin eruption with edema of both lids. He readily responded to treatment with an antihistamine drug. On further questioning the patient recalled having experienced a similar reaction when penicillin had been administered elsewhere by the parenteral route. Thus it was found that large doses of crystalline penicillin might be safely instilled into the antral cavity

* Obtained from the U. S. Department of Agriculture.

† Kindly supplied by The Schering Corporation.

provided the usual precautions in respect to allergic reactions to the drug were observed.

As may be noted from Table I, a significant blood penicillin level was present in 24 out of 26 cases with chronic suppurative sinusitis following the intra-antral instillation of 200,000 units of the crystalline product. In every instance except one (Patient No. 25) the blood level was uniformly higher after the addition of hyaluronidase to the penicillin solution. In general, the blood levels were found to be about 2 to 3 times greater than those found without its use. Furthermore, it is of interest that patients No. 19 and No. 21 in whom no evidence of absorption could be demonstrated, showed significant levels after the addition of hyaluronidase. These findings suggest that hyaluronidase increases diffusion and penetration of penicillin through the diseased antral mucosa.

Similar findings to those noted above were obtained in subjects with non-diseased mucous membranes (Table II). A significant level was found in the blood of all 5 subjects after the antral instillation of penicillin and in each patient tested, the addition of hyaluronidase resulted in higher levels.

Although we were not primarily concerned in this study with the evaluation of the clinical effectiveness of large doses of crystalline penicillin supplemented by hyaluronidase in the treatment of chronic suppurative disease of the maxillary sinus, it is of interest that marked clinical improvement occurred in some of the patients.

Summary. The instillation of 200,000 units of crystalline penicillin G into the diseased and normal paranasal antrum is well tolerated and except for the development of an allergic reaction in one patient, was without any adverse effect. In 24 out of 26 patients with chronic suppurative disease of the sinuses and in all 5 normal subjects, a significant penicillin level in the blood was found after the intra-antral instillation.

In both groups, the addition of hyaluronidase to the instilled penicillin resulted in even higher blood levels than those found without its use, with one exception. In two patients in whom no blood level could be demonstrated, the addition of hyaluronidase resulted in a significant concentration of penicillin in the blood. It is postulated that the increased blood penicillin level following hyaluronidase is due to greater diffusion and penetration of the penicillin as a result of the spreading action of hyaluronidase.

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Tuberculin Reaction. III. Transfer of Systemic Tuberculin Sensitivity with Cells of Tuberculous Guinea Pigs.*

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In spite of many years of research by numerous investigators the passive transfer of tuberculin sensitivity was not accomplished with any degree of certainty or regularity until the important work of Chase[1] whose technic has provided a new means of exploring many aspects of this highly important type of sensitivity.†

In previous publications,[2,3] we have pre-

* This investigation was supported by a grant from the United States Public Health Service.

1 Chase, M. W., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 134.

† The term sensitivity is used throughout the present article in preference to the more commonly used term hypersensitivity.

sented experimental evidence which indicates that the tuberculin reaction is the result of the interaction of a sessile antibody with specific antigen. In this work[2] the observation of Chase[1] that cutaneous tuberculin sensitivity can be passively transferred with the cells of induced peritoneal exudates of non-tuberculous donor guinea pigs sensitized with heat-killed tubercle bacilli was confirmed by the use of tuberculous donors. Another observation was that the *in vitro* effects of tuberculin in suppressing the initial migration of leukocytes from splenic explants of tuberculous guinea pigs is markedly reduced by specific desensitization.[3] In addition the results of preliminary trials were presented which indicated that homologous passive transfer of systemic tuberculin sensitivity can be accomplished in the guinea pig with cells of peritoneal exudates of tuberculous donors.[2]

The results given in the present report confirm and extend the observations of these preliminary trials.

Although the systemic tuberculin reaction was recognized as early as the local cutaneous reaction,[4,5] and is still used in the standardization of Old Tuberculin, it is not employed as widely as the cutaneous reaction for determining tuberculin sensitivity in experimental tuberculosis of laboratory animals. It is unfortunate that the systemic reaction commonly involves the sacrifice of these animals, since it is probably a more reliable measure of tuberculin sensitivity than the cutaneous reaction which is observed to be non-specifically affected by many factors such as age,[6,7] general health and intercurrent disease.[8–11]

Koch[4,5] observed that fatal systemic tuberculin shock in the guinea pig is so characteristic that there is little reason to confuse it with other types of shock. The principal features which Koch noted to characterize the reaction were the delayed onset, the protracted course with extreme malaise and the focal reactions at the site of tuberculous lesions.

In his studies on systemic tuberculin shock in the guinea pig Weinzirl[12] was also much impressed by the inactivity of the animals, the limpness of their muscles, and the anorexia and general prostration they exhibited. He proposed that the experimental provocation of the systemic tuberculin reaction by the intraperitoneal injection of tuberculin be called the "systemic test".

The systemic tuberculin reaction has been used in the past as an index of sensitivity in many of the numerous attempts that have been made to accomplish homologous and heterologous passive transfer of tuberculin sensitivity in laboratory animals and man.[13–23] In these attempts a variety of transfer materials were used such as serum, whole blood, exudates, transudates, and organ mashes. The

2 Kirchheimer, W. F., and Weiser, R. S., PROC. SOC. EXP. BIOL. AND MED., 1948, **66**, 166.

3 Kirchheimer, W. F., and Weiser, R. S., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 407.

4 Koch, R., *Dtsch. Med. Wchnschr.*, 1890, **16**, 1029.

5 Koch, R., *ibid.*, 1891, **17**, 101.

6 Freund, J., *J. Immunol.*, 1927, **13**, 285.

7 Freund, J., *ibid.*, 1929, **17**, 465.

8 Pilcher, J. D., *Am. Rev. Tuberc.*, 1930, **21**, 669.

9 Mitchell, A. G., Wherry, W. B., Eddy, B., and Stevenson, F. E., *Am. J. Dis. Child.*, 1928, **36**, 720.

10 Bloomfield, A. L., and Mateer, J. G., *Am. Rev. Tuberc.*, 1919, **3**, 166.

11 v. Pirquet, C. E., *Arch. Int. Med.*, 1911, **7**, 259, 383.

12 Weinzirl, J., *Tubercle*, 1931, **12**, 488.

13 Friedemann, V., *Münch. Med. Wchnschr.*, 1907, **49**, 2414.

14 Bauer, J., *ibid.*, 1909, **56**, 1218.

15 Roepke, *Z. f. Medizinalbeamte*, 1910, No. 5, p. 149.

16 Bail, O., *Z. f. Immunitatsforschg.*, 1909, Orig. IV, 470.

17 Joseph, K., *Beitr. z. Klin. der Tuberk. von Bauer*, 1910, *Bd.* **17**, 461.

18 Yamanouchi, T., *Zentralbl. f. Bakt.*, 1909, Part I, **44**, 434.

19 Yamanouchi, T., *Comp. Rend. Soc. Biol.*, 1909, **66**, 531.

20 Onaka, M., *Beitr. z. Klin. der Tuberk. von Bauer*, 1910, Orig. VII, 507.

21 Kraus, R., Loewenstein, E., Volk, R., *Zentralbl. f. Bakt.*, 1911, I, **50**, 361.

22 Massol, L., Breton, M., and Bruyant, L., *Comp. Rend. Soc. Biol.*, 1913, **74**, 185.

23 Selter, H., *Z. f. Immunitatsforschg.*, 1921, **32**, 325.

results of these various experiments were irregular and inconclusive.

Methods. In the present investigation the guinea pigs employed as cell donors weighed between 650 and 800 g and were sensitized 5-9 weeks prior to use by the intraperitoneal injection of approximately 5 mg of living *Mycobacterium tuberculosis* var. *bovis* of the B.C.G. strain. Only those animals showing necrotic reactions to the intradermal injection of 0.1 ml of 1:100 O.T. were used as donors. Cell exudates were induced in the donor animals as outlined by Chase[1] by the intraperitoneal injection of 25 to 30 ml of paraffin oil.‡ The animals were killed 48 hours later and the cells collected and washed by centrifugation in heparinized guinea pig serum—Tyrode solution in the manner described by Chase.[1]

The recipients were large, healthy male guinea pigs each of which was injected intraperitoneally with the washed, pooled cells from 6 donors. Each recipient was challenged 48 hours after receiving the donor cells by the intraperitoneal injection of 2 ml of O.T. (1X International Standard) diluted with 8 ml of 0.85% NaCl.§

The controls consisted of recipients given intraperitoneal injections of comparable quantities of cells from (1) normal donors, (2) donors sensitized 5 to 9 weeks previously with 5 mg of living *Mycobacterium smegmatis,* (these animals gave 3+ cutaneous reactions to smegmatis tuberculin 1:100) (3) donors injected 5 to 9 weeks previously with powdered quartz and (4) tuberculous donors desensitized by the repeated subcutaneous injection of increasing doses of O.T. in the manner previously described.[3] These controls were challenged with O.T. in the manner described above. An additional control group consisted of recipients given the cells of sensitive donors and challenged with a dose of diluted glycerine broth comparable to the concentration of these substances present in the O.T. preparation used.

The peritoneal cells of the sensitive donors and the control groups, including desensitized animals, were examined by the neutral red supravital method as outlined previously.[2] A large percentage of the cells of all of the preparations were found to be living.

Except for the development of slight abdominal tenderness in an occasional animal, all recipients appeared to remain physically normal during the 48 hour period prior to the challenging dose of shock test substance. Their rectal temperatures remained at normal levels.

Results. The results of the principal experiments are recorded in Table I. They show that of the 8 recipients of cells from sensitive animals, 5 died of tuberculin shock in from 20 to 50 hours, and 3 were severely shocked but recovered. Among the control groups one animal developed tuberculin shock.

A mild, but transient discomfort was observed in most of the animals immediately following the challenging injection of tuberculin. This was apparently due to the glycerine, since the same effect was observed following injection of normal animals with similar dilutions of glycerine and likewise in the group of recipients of the cells of sensitive animals challenged with diluted glycerine broth.

One of the recipients of cells of desensitized tuberculous donor animals developed mild symptoms of tuberculin shock and recovered. Another animal of this group showed no symptoms of shock following the challenging dose of O.T. but suddenly died at the 52nd hour. There was no indication at autopsy as to what the cause of death may have been. It seems unlikely that it was due to tuberculin shock since the characteristic symptoms of tuberculin shock were lacking.

All of the recipients of the cells of sensitive donors developed typical systemic tuberculin shock which usually began after an interval of about 3 hours and became severe by 4 to 5 hours. The first evidence of shock was the development of muscle weakness ruffled hair and increased respiration. In all instances a marked drop in body temperature

‡ The mineral oil used was Bayol F. distributed by Stanco Incorporated, 2 Park Avenue, New York City.

§ The Old Tuberculin used in these experiments was kindly supplied by the Lederle Laboratories of Pearl River, N. Y.

TABLE I. Passive Transfer of Systemic Tuberculin Sensitivity with Cells of Induced Peritoneal Exudates of Tuberculous Guinea Pigs.

Recipient No.	Preparation of donors	Sensitizing dose of cells in ml	Dose of challenging material	Reaction of animal
1	B.C.G. sensitized	.5	10.0 ml diluted O.T.	Died at 30 hrs.
2	" "	.5	" " " "	" " 21 "
3	" "	1.0	" " " "	" " 21 "
4	" "	.5	" " " "	In severe shock at 4 hrs, fully recovered at 30 hrs.
5	" "	.5	" " " "	Died at 20 hrs.
6	" "	.5	" " " "	Severe shock at 4 hrs, fully recovered after 48 hrs.
7	" "	.8	" " " "	Severe shock at 5 hrs, fully recovered after 48 hrs.
8	" "	.5	" " " "	Died at 50 hrs.
Controls				
1	*M. smegmatis* sensitized	.5	" " " "	No symptoms.
2	B.C.G. sensitized and desensitized	1.8	" " " "	Slight symptoms shock 4 hr, fully recovered 10 hr
3	" " "	.6	" " " "	No symptoms.
4	" " "	.7	" " " "	No symptoms, but died suddenly after 52 hrs.
5	Quartz treated	.5	" " " "	No symptoms
6	" "	.8	" " " "	" "
7	Normal	.5	" " " "	" "
8	"	1.2	" " " "	" "
9	B.C.G. sensitized	.9	10.0 ml diluted glycerine broth	" "
10	" "	.5	" " " "	" "

occurred, which in fatal cases went as low as 34°C just prior to death. In fatal cases the severity of shock increased progressively until death and marked abdominal tenderness was observed to develop in many of the animals during the late stages of shock. In those that survived, recovery from the early symptoms of shock usually began about the 20th hour and continued gradually with the result that shock symptoms disappeared entirely by about the 48th hour.

All of the animals that died were promptly autopsied. A small amount of clear yellowish and at times sanguinous fluid was commonly found in the peritoneal cavity. The omentum was rolled up and the omental folds adherent. A small amount of fibrinous deposit was occasionally present on the surface of the liver. The mesenteric vessels appeared to be engorged and a pronounced reddish discoloration of the walls of the small intestines was found with great regularity. The liver was large and distended with blood. Bacteriological examinations of the peritoneal fluid by smear and culture were negative in all instances. There were no other abnormal findings.

Summary. The results of the present investigations establish that systemic tuberculin sensitivity can be passively transferred in the guinea pig with the cells of induced peritoneal exudates of tuberculous donors. The systemic shock produced in recipients passively sensitized in this manner is of the typical delayed tuberculin type. These findings supplement the observation of Chase[1] that cutaneous tuberculin sensitivity can be passively transferred in the guinea pig with the cells of induced peritoneal exudates of donors sensitized with heat-killed tubercle bacilli by providing additional evidence that the sensitivity transferred is of the tuberculin type. In a limited number of trials it was also found that desensitization of tuberculous donors with tuberculin tends to abolish the capacity of their peritoneal cells to passively transfer tuberculin sensitivity. If this observation is confirmed it will provide strong evidence that desensitization results from the saturation of fixed cellular antibody with antigen.

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Carious Lesions in Cotton Rat Molars. II. Effect of Removal of Principal Salivary Glands.*

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An appreciable increase in susceptibility to dental caries has been observed in albino rats which were maintained on coarse particle diets after the removal of some or all of the principal salivary glands.[1-4] Similarly, a substantial increase in susceptibility to tooth decay has been observed in Syrian hamsters after the extirpation of the major salivary glands.[5] The diets used in these investigations were composed primarily of natural foodstuffs. In the present experiments, the principal salivary glands of weanling cotton rats were removed prior to periods of maintenance on caries-producing, purified rations to determine if this procedure would result in a greater number and extent of carious lesions and a different distribution of the lesions with respect to the various tooth surfaces.

Experimental. The cotton rats to be used in this experiment were separated from their mothers at 14 days of age and divided into two groups, equal with respect to parentage, sex and weight. The individuals in the one group were kept as intact controls, while the principal salivary glands of the cotton rats in the other group were removed under ether anesthesia. After the operation, each animal was placed in an individual wire-bottom cage with access to water and to either of 2 caries-producing purified rations *ad libitum*. Half of the cotton rats in each of the experimental and control groups were provided with ration 100.[6] The basal composition of this ration was: sucrose 67%, casein 24%, salt mixture 4% and corn oil 5%, supplemented with adequate amounts of the fat-soluble vitamins, the B vitamins and liver concentrates to permit normal growth. The other half of the cotton rats in each of the intact and operated groups was fed a caries-producing ration of slightly lower caries-producing properties, ration 130, which was a slight variant of ration 100 attained through the isocaloric replacement of 18 of each 67 parts of sucrose by 8 parts of lard by weight. When rations comparable to ration 130 were fed after the development of the teeth was largely complete, the carbohydrate-protein-fat distribution has been shown previously to be borderline in its ability to reduce the susceptibility of the molar teeth of cotton rats to the initiation and development of carious lesions during a 14 week experimental period.[7-9] At the end of 12 weeks after desalivation, all cotton rats were sacrificed and the number and extent of the carious lesions determined for each animal by previously described criteria.[10,11]

Results. The average weight gain during

* This project was supported in part by a grant from the Sugar Research Foundation, Inc., New York. We are indebted to Merck and Co., Rahway, N. J., for gifts of the B-complex vitamins used in this study.

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TABLE I.
Effect of Extirpation of Principal Salivary Glands on Rate of Growth and Incidence and Extent of Carious Lesions in the Cotton Rat.

Ration	Condition	No. of rats	Avg gain in g	Avg No. of carious lesions	S.E.M.*	C.R.†	Avg extent of carious lesions	S.E.M.*	C.R.†
100	Normal	7	50	22.1	2.0		50+	8+	
						5.1			5.6
"	"Desalivated"	9	28	33.9	1.1		109+	7+	
130	Normal	4	54	17.0	2.1		31+	9+	
						3.1			3.0
"	"Desalivated"	5	42	25.0	1.6		63+	6+	

* S.E.M., Standard error of means.
† C.R., Critical ratio.

the experimental period and the average number and extent of carious lesions developed in intact cotton rats and littermates from which the major salivary glands were removed are presented in Table I. Those cotton rats which had had their principal salivary glands removed prior to 12 weeks maintenance on ration 100 had a 53% higher average number of carious lesions and a 110% higher average extent of carious lesions than their intact littermates which had been fed ration 100 for the same period. Likewise, the desalivated animals which had been maintained on ration 130 had a 47% higher average number of carious lesions and a 100% increase in the average extent of carious lesions over that demonstrated by their normal littermates which had received ration 130. These increases in average number and average extent of carious lesions were sufficiently large to be judged statistically significant for both rations. As has been previously demonstrated, the intact cotton rats which were maintained on ration 130 for the 12 week experimental period had a slightly lower average number and average extent of carious lesions than their normal littermates which were maintained on ration 100. Likewise, the desalivated cotton rats which were maintained on ration 130 had an appreciably lower average number and average extent of carious lesions than their desalivated littermates which were maintained on ration 100 throughout the experimental period. Thus the post-developmental caries-retarding effect of ration 130 occurred not only in normal but in desalivated cotton rats and to approximately the same degree in both the intact and operated animals.

Despite the fact that the average number and the average extent of the carious lesions were significantly higher in desalivated cotton rats than in their intact littermates, almost all carious lesions in intact and operated animals developed in the sulci of the molars except for a few lesions on the proximal surfaces of the molars. The increased occurrence of carious lesions in desalivated cotton rats was a result of the involvement of a higher percentage of the sulci and of the proximal surfaces than occurred in normal animals. No carious lesions were found on the smooth surfaces nor on the occlusal surfaces in either intact or desalivated animals.

At the end of the 12 week experimental period, the crowns of the molars in all 4 quadrants of the desalivated cotton rats which had received ration 100 had been considerably reduced in volume by tthe carious processes and the subsequent fracture of undercut cusps. The remaining areas of the smooth surfaces were heavily covered with food debris but no gross lesions were observable under this

material by the methods of detection used. In the cotton rats fed ration 130 much less of the molar crowns had been destroyed by the carious processes; thus the size of the smooth surfaces had not been reduced as much but were as heavily covered with food debris. Again no carious lesions were observed on the smooth surfaces. Thus the relative distribution of carious lesions on the various tooth surfaces in the desalivated cotton rat appeared to be identical with that in the normal cotton rat.

The rate of growth was approximately the same for normal cotton rats receiving rations 100 and 130. The rate of growth for the desalivated cotton rats was appreciably less than for their intact littermates. This reduction in rate of growth was greatest in the animals which received ration 100 throughout the experimental period.

Summary. The removal of the principal salivary glands of cotton rats prior to maintenance on purified, caries-producing rations resulted in a significantly greater average number and average extent of carious lesions than was observed in their intact littermates maintained under otherwise identical experimental circumstances. No alteration in the relative distribution of the lesions with respect to the various tooth surfaces resulted from the removal of the major salivary glands.

16839 P

Inhibition of the Appearance of Phenol Red in Frog Kidney Tubules *in vitro*.

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4′-Carboxyphenylmethanesulfonanilide (caronamide) inhibits concentration of phenol red in the lumina of the tubules of frog kidney slices.† This observation suggested that it might be of interest to study the activity of various other substances as inhibitory agents. A number of compounds have been studied. These have been mainly carboxylic acids; however, the present communication is concerned only with derivatives of cinchoninic acid. A recent study in this laboratory has revealed that certain derivatives of cinchoninic acid inhibit a water diuresis in the dog.[1] Some of these compounds have been found to increase uric acid excretion in man.[2-4] The experiments described in this report show that the appearance of phenol red color in the lumina of the tubules in frog kidney slices is inhibited by certain of these substances.‡

Procedure. A modification of the method of Forster has been used.[5] Both kidneys of a frog (*Rana pipiens*) were removed and sliced transversely into sections approximately 200 microns in thickness. The slices were mixed and distributed to 12 small Petri dishes each containing 5 ml of the solution described by Forster.[5] The concentration of phenol red used was 0.03 millimoles per liter. The substances being tested were dissolved in this

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† Dr. E. K. Marshall, Jr., in conjunction with Dr. Roy P. Forster, made this observation on the fish kidney (flounder) in Maine in the summer of 1947.

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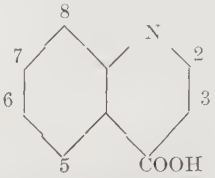
‡ We wish to thank Dr. K. C. Blanchard for supplying the cinchoninic acid derivatives.

5 Forster, Roy P., *Science*, 1948, **108**, 65.

TABLE I.

Effect of Cinchoninic Acid Derivatives on the Concentration of Phenol Red by Frog Kidney Tubules *in Vitro*.



Substituent	Min. inhibitory conc. Cinchoninic derivative mM/1	Caronamide mM/1	Ratio*
None	2	.5	.25
2-Hydroxy	>2	.25	< .12
2-Hydroxy-3-methyl	>2	.3	< .15
2-Hydroxy-3-phenyl	4	.25	.06
2-Methyl	1	.125	.12
3-Hydroxy-2-methyl	0.1	.3	3
3-Carboxy-2-methyl	>4	.125	< .03
2-Phenyl	.125	.125	1
3-Hydroxy-2-phenyl	.03	.125	4
6-Methoxy	.08	.15	2

* The ratio of the minimal inhibitory concentration of caronamide to the minimal inhibitory concentration of the unknown.

solution before the kidney slices were placed therein. Oxygen was continuously bubbled through the solution in each dish by a small jet made from a 27 gauge hypodermic needle. These 12 jets were fed from a manifold.

Caronamide was used as a standard of comparison. Each experiment included three concentrations of caronamide and two controls containing no drug. In all cases the concentrations of the substances being tested were varied by a factor of two. To avoid subjective errors the minimum concentration of drug which completely prevented the appearance of phenol red color in the tubule lumina was used to evaluate effectiveness. Decrements in drug concentration below the minimal inhibitory level resulted in graded increments in the amount of color seen in the tubule lumina. The minimal inhibitory concentration of caronamide varied somewhat from one experiment to another.

Results and discussion. In Table I the inhibitory concentrations of various substances are compared with the concentration of caronamide necessary to produce the same effect in the same experiment. Only the 3-hydroxy-2-methyl, the 3-hydroxy-2-phenyl and the 6-methoxycinchoninic acids were more active than caronamide, while the 2-phenyl derivative (cinchophen) was equally active.

The desirability of comparing these results with results obtained *in vivo* in mammals is obvious. We have therefore begun such a study. In preliminary experiments, it appears that some members of this group depress the clearance of phenol red in the dog.

Summary. Cinchoninic acid and nine of its derivatives have been studied with respect to the inhibition of the appearance of phenol red color in the lumina of the tubules of frog kidney slices *in vitro*. Three of these have been found more active in this respect than is caronamide and one equally active.

16840 P

Subcortical Centers as Pacemakers of Cortical Activity.*

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In continuation of a previous investigation[1] in which the effect of anoxia and asphyxia on normal and convulsive potentials of the cortex was studied, experiments were undertaken to determine the hypothalamic-cortical relations under these conditions. All experiments were performed on curarized "Dial" cats (intocostrin, artificial respiration) in which after exposure of the brain cortical, hypothalamic, and thalamic potentials were recorded with mono- and bipolar silver electrodes. An Offner inkwriter and amplifier were used. Subcortical electrodes were inserted with the Horsley-Clarke apparatus. Strychnine pledgets served to elicit local cortical spikes; this drug was also injected either into the hypothalamus or intravenously.

In experiments in which the cortex was strychninized the results were similar to those discussed in the preceding paper. However, if strychnine was injected intravenously a series of very regular small spikes appeared synchronously in cortex and hypothalamus at a time when due to the asphyxia normal and convulsive potentials had either completely disappeared or were greatly reduced in amplitude and frequency. This burst of activity lasted in the majority of observations for 16 to 25 seconds although in some instances it was as brief as 6 or as long as 34 seconds. The frequency of discharge was variable. In general the amplitude declined greatly as the frequency increased, the maximum being about 14 to 18 per second.

Since the synchrony of discharge suggested a subcortical pacemaker strychnine was injected into the hypothalamus and in other instances into several nuclei of the thalamus. Although heavy strychninization of the hypothalamus may lead to appearance of spikes in the cortex[2] injection of this drug into a minute area of the hypothalamus induced local spiking but did not interfere with normal cortical activity and the asynchrony existing between different cortical areas. Asphyxia caused under these conditions likewise the appearance of synchronous spikes in various cortical areas and in the hypothalamus (Fig. 1). These potentials were larger in monopolar than in bipolar recordings. Cathode ray oscillograms showed that they consisted of bipolar spikes. This phenomenon was reversible and could be repeated many times in the same preparation. As time progressed the spikes appearing in the injected area became less in frequency and amplitude and then disappeared. Even at this stage the synchronous discharge could be induced in asphyxia. When some time later synchrony was no longer elicited it could be reinvoked after reinjection of strychnine into the hypothalamus.

The synchronous discharges discussed in this paper were recorded bilaterally in the cortex after unilateral injection of the hypothalamus or thalamus with strychnine. They were likewise found in contralateral thalamic nuclei. This phenomenon occurred in asphyxia as well as in anoxia (inhalation of nitrogen or nitrogen-air mixtures). It is probably related to the synchronous spindles which van Harreveld[3] observed in the cortex after prolonged asphyxia. It is suggested that:

1. Hypothalamic and closely related thala-

* Aided by a grant of the Office of Naval Research.

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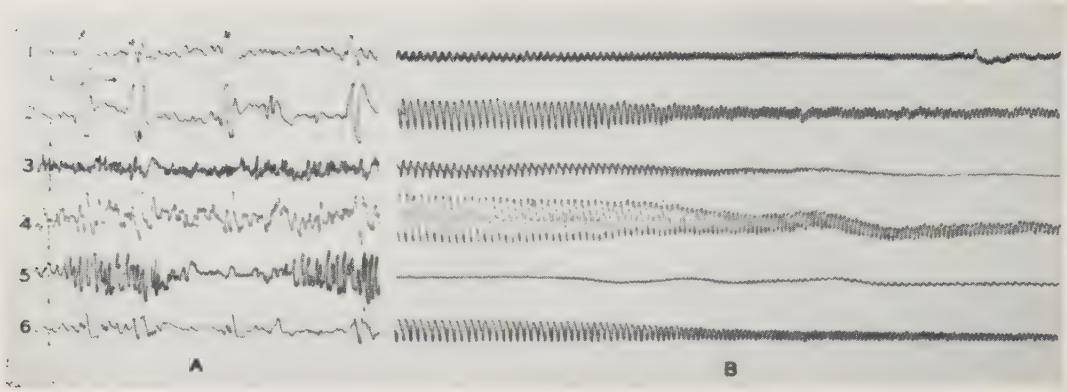

FIG. 1.

Dial cat. Right hypothalamus injected with 0.03 mg strychnine. A, control; B, during asphyxia, immediately after normal and convulsive potentials had been abolished. Note asynchrony in control and synchronous discharge in asphyxia.

1. Left ventrolateral thalamus (monopolar).
2. Right hypothalamus (monopolar) injected with strychnine.
3. Left motor cortex (bipolar).
4. Same site, monopolar.
5. Left auditory cortex (bipolar).
6. Same site, monopolar.

Calibration 100 microvolts and 1 second.

mic nuclei[2,4] do not act as pacemakers of cortical activity as long as the excitability of the cortex is unchanged.

2. In conditions such as asphyxia and anoxia which lead to a relative predominance of hypothalamic excitability the discharge of these subcortical nuclei determines the qualitative and quantitative activity in thalamus and cortex and leads to periods of complete synchrony of hypothalamic, thalamic and cortical activity.

[4] Gellhorn, E., and Ballin, H. M., *Am. J. Physiol.*, 1946, **146**, 630.

16841

Relation of Iodinated β-amylose (Tridine) to Some Thyroid Functions.

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The appearance of iodinated β-amylose, "Tridine",† a compound of carbohydrate and iodine, suggested a biological assay to determine whether it behaves like an inorganic salt of iodine or like other iodinated organic compounds such as thyroxin or thyroglobulin.

Methods. White albino rats were used throughout the experiment. All animals received ground dog biscuit‡ and water *ad libitum* for 5 to 7 days before any experiment was begun. During the experimental period the control group remained on this diet while

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† According to the manufacturer, The Clairmint Chemical Co., 7 Lackawanna Avenue, Newark 2, N. J. "Tridine" is iodinated β-amylose and contains approximately 20% of iodine by weight. The Clairmint Chemical Co., defrayed part of the expense of this investigation.

‡ Purina Laboratory Chow.

TABLE I.
Effects of Tridine and Thyroxine on Thiouracil-induced Hypothyroidism.

	Organ wt per 100 g body wt					Metabolic rate*	
	Pituitary mg	Thyroid mg	Adrenals mg	Kidneys g	Liver g	0 days	10 days
Group 1 (6)† Control	5.4 ±0.37	9.0 ±0.73	30.3 ±1.22	0.68 ±0.019	4.0 ±0.16	77.0	81.0 + 4.2%
Group 2 (6) 0.1% Thiouracil	6.3 ±0.33	15.9 ±0.51	26.0 ±1.14	0.75 ±0.023	5.2 ±0.25	82.6	67.0 —18.9%
Group 3 (6) 0.1% Thiouracil + 3% Tridine	6.5 ±0.36	13.2 ±0.73	26.5 ±0.73	0.77 ±0.080	4.1 ±0.26	76.8	64.2 —16.4%
Group 4 (6) 0.1% Thiouracil + Thyroxine	6.2 ±0.15	9.9 ±0.66	28.1 ±1.77	—0.74 ±0.016	3.7 ±0.14	—	79.3

* Calories/24 hr/kg¾.
† No. of animals.

the other groups received their medication in addition as a dietary supplement. The food intake was recorded and the animals were weighed every other day. The animal room temperature was maintained at 27° ±1°C.

At the beginning and termination of each experimental period the resting, fasting (24 hr) metabolic rates were determined by the use of a modified Haldane apparatus.[1] By the use of the preliminary fast and an alternate carbon dioxide absorbing jar which was switched on only when the rats were inactive it was possible to approximate the basal metabolic rate. The metabolic rates were calculated as calories per 24 hours per kilogram to the three-fourths power.

At the end of each experimental period, the animals were sacrificed and the pituitary, thyroids, adrenals, liver, and kidneys weighed and computed as weight of organ per hundred grams of body weight. Histological sections were taken when indicated and stained with hematoxylin and eosin.

Results. The mean results with their standard errors are given in the tables. Any change that could occur by chance in only 5% or less of the trials is considered significant.

Experiment I. Twenty-four adult (150 g) female rats were used in this series. After a preliminary period of 7 days, the metabolic rates were determined and medication begun. For this purpose they were divided into 4 groups of 6 animals each. Group 1 served as controls, Group 2 received 0.1% thiouracil in the food, Group 3 received 3% tridine and 0.1% thiouracil in the food, and Group 4 received 0.1% thiouracil in the food and 0.02 mg of *l*-thyroxine injected subcutaneously every other day. After 10 days a final metabolic rate determination was made and the animals sacrificed, the organs weighed, and histological sections taken of the thyroid and kidney of all animals. The data are assembled in Table I. Histologically, all the animals showed normal renal tissue. Group 1 showed normal thyroid tissue. Group 2 showed changes in the thyroid typical of thiouracil medication, namely, nearly complete disappearance of the colloid substance together with marked hyperplasia of the epithelial cells with a change from the cuboidal to the high columnar form. In Group 3 this picture was modified so that colloid was present and more abundant than in the previous group, but it was still subnormal in amount. There was also less evidence of epithelial hyperplasia, but it was present to a slight degree. Group 4 showed thyroid sections which were identical with the control group.

Tridine had no significant effect on the lowered metabolic rate of thiouracil-treated

[1] Anderson, J. T., and Nasset, E. S., *J. Nutrition*, 1948, **36**, 703.

TABLE II.
Effect of Tridine on Thyroxine-treated Adult Rats.

	Organ wt per 100 g body wt					Metabolic rate*	
	Pituitary mg	Thyroid mg	Adrenals mg	Kidneys g	Liver g	0 days	10 days
Group 5 (6)† Thyroxine	7.7 ±0.54	9.1 ±0.51	29.6 ±1.23	0.82 ± .014	4.2 ±0.11	79.8	91.5 +14.7%
Group 6 (6) Thyrox. + 1% Tridine	7.1 ±0.42	9.3 ±0.78	31.5 ±1.67	0.94 ±.030	4.5 ±0.11	79.0	90.5 +14.6%
Group 7 (6) Thyrox. + 3% Tridine	6.7 ±0.42	10.8 ±1.61	30.1 ±3.39	0.92 ±0.040	4.5 ±0.28	76.5	83.8 + 9.5%
Group 8 (6) Thyrox. + 5% Tridine	7.5 ±0.19	10.8 ±0.30	32.8 ±3.65	0.95 ±0.074	5.3 ±0.66	77.0	86.1 +11.8%

* Calories/24 hr/kg¾.
† No. of animals.
All rats received 0.02 mg thyroxine subcutaneously every other day.

rats. (The standard error of the metabolic rate determination is about 2.5% of the total value). The thyroid glands of Group 3 were significantly smaller than those in Group 2 (thiouracil alone) but it will be shown later that this is not a specific effect of tridine.

Experiment II. Twenty-four adult (190 g) female rats were divided into 4 groups of 6 rats each and after an acclimatization period of 5 days, metabolic rates were determined and medication begun. All received 0.02 mg of thyroxine subcutaneously every other day. Group 5 received stock diet alone, while Group 6 received 1% tridine, Group 7 received 3% tridine, and Group 8 received 5% tridine, all mixed with the stock diet. After 10 days another metabolic rate determination was made, the animals sacrificed, and organ weights recorded. No histological sections were taken. Results are set out in Table II.

This experiment was designed to test the possible effect of feeding tridine (1, 3 and 5% of the diet) on hyperthyroidism produced by injection of thyroxine. The only significant change in all of these observations was an increase in the size of the thyroids of Group 8. All of the other organs as well as the energy metabolism were not significantly altered by tridine treatment. It is evident, therefore, that tridine in the dosages employed does not exhibit any marked antagonism to thyroxin.

Experiment III. It was of some interest to determine what influence the feeding of tridine might have on the growth of young animals. Twenty rats of both sexes about 4 weeks old were divided into 4 groups of 5 rats each and after a preliminary period of 5 days, the metabolic rates were determined and the animals started on the medication. Group 9 received stock diet, Group 10 received 1% tridine, Group II received 3% tridine and Group 12 received 5% tridine, all mixed with stock diet. After 18 days, the metabolic rates were again determined, the animals sacrificed, organ weights recorded, and histological sections of the thyroid gland taken. Table III shows the organ weights and the metabolic rates. Table IV shows weight gains, food consumption and efficiency of food utilization. There were no fatalities in any of the groups.

Histologically, the thyroid glands showed little deviation from the normal. There was slightly more colloid material present in those rats receiving 3% and 5% tridine than in the control and the 1% tridine group. There was, however, no evidence of epithelial hyperplasia. It is evident from Table III that the thyroid in Group 10 was significantly larger than the control (Group 9). The adrenal glands and the liver were significant-

TABLE III.
Tridine as a Dietary Supplement for Young Rats.

	Organ wt per 100 g body wt					Metabolic rate*	
	Pituitary mg	Thyroid mg	Adrenals mg	Kidneys g	Liver g	0 days	18 days
Group 9 (5)† Control	4.7 ±0.11	8.5 ±0.81	20.0 ±0.50	0.93 ±.028	6.2 ±0.11	98.0	88.6 — 9.6%
Group 10 (5) 1% Tridine	5.1 ±0.49	12.3 ±0.67	22.2 ±0.69	0.94 ± .032	6.7 ±0.19	99.5	88.1 —11.5%
Group 11 (5) 3% Tridine	4.6 ±0.24	9.9 ±0.96	23.8 ±0.47	0.97 ±.021	7.3 ±0.23	100.0	89.9 —10.1%
Group 12 (5) 5% Tridine	5.6 ±0.71	10.0 ±1.58	29.4 ±4.75	1.05 ±.104	6.7 ±0.71	99.0	80.3 —19.0%

* Calories/24 hr/kg¾.
† No. of animals.

TABLE IV.
Food Utilization in Young Rats as Affected by Tridine Supplement in the Diet.

	Avg wt		Avg gain g	Avg food intake g	G food eaten per g wt gained
	0 days g	16 days g			
Group 9 Control	56.0	136.4	80.4	200.9	2.5
Group 10 1% Tridine	53.6	118.4	64.8	172.4	2.7
Group 11 3% Tridine	50.8	104.6	53.8	147.6	2.7
Group 12 5% Tridine	50.8	76.4	25.6	112.9	4.5

ly larger in Groups 10 and 11. The energy metabolism was depressed significantly in Group 12 as compared with the other groups which exhibited only the normal decline in metabolic rate associated with maturation. Table IV shows that with all dosages of tridine there was a loss of appetite and a corresponding failure to gain weight. In Group 12 weight gained per gram of food eaten was very low and taken together with the depressed metabolism may be regarded as a sign of toxicity with possible impaired digestion and absorption of food. There was slight but inconclusive histological evidence of increased colloid production induced by tridine. There was, however, no increase in the metabolic rate to corroborate this finding. The maximum tridine intake was approximately 5.5 g per kilo per day.

Experiment IV. Ten young (125 g) rats were used, 4 males and 6 females, equally divided into the 2 groups of 5. After a preliminary period of 5 days, metabolic rates were determined and medication was begun. Both groups received 0.1% thiouracil in the stock diet and in addition Group 13 received 3% tridine and Group 14 received 0.77% potassium iodide in the diet. These percentages represent equivalent amounts of iodine. After ten days a final metabolic rate was determined, the animals sacrificed, their organs weighed, and histological sections made of the thyroid glands. Results are recorded in Table V. Histological sections showed a modified thiouracil effect in both cases, the picture being similar to that of Group 3 in Experiment 1. In both groups there was a partial reversal of the thiouracil effect. There are no signifi-

TABLE V.
Comparison of Equivalent Amounts of Tridine and KI in Thiouracil-treated Rats.

	Organ wt per 100 g body wt					Metabolic rate*	
	Pituitary mg	Thyroid mg	Adrenals mg	Kidneys g	Liver g	0 days	10 days
Group 13 (5)†	5.4 ±0.32	13.7 ±1.13	18.8 ±2.55	0.99 ± .062	5.7 ±0.33	86.3	73.5 —14.8%
Group 14 (5)	5.7 ±0.73	12.5 ±1.78	18.9 ±2.57	0.86 ± .032	4.9 ±0.26	79.6	72.4 —10.0%

Group 13—0.1% Thiouracil in diet + 3% Tridine.
Group 14—0.1% Thiouracil in diet + 0.77% KI (≏ 3% Tridine in I).
* Calories/24 hr/kg$^{3/4}$.
† No. of animals.

cant differences between these groups in organ weights, energy metabolism or histological appearance of the thyroid gland. It is concluded that tridine exerts no unique effect which cannot be accounted for on the basis of its iodine content.

Discussion. It is evident from the results that tridine, in the animal body, behaves like an equivalent amount of inorganic iodine. Like KI,[2] tridine is capable of preventing partially the thyroid hyperplasia which accompanies thiouracil feeding. The low metabolic rate of thiouracil-treated rats is unaffected by feeding tridine as 3% of the diet. Moderate hyperthyroidism produced by the administration of thyroxin does not seem to be influenced significantly by the addition of tridine as a dietary supplement in concentrations of 1, 3 and 5%. From the fact that tridine is approximately equivalent, on the basis of iodine content, to KI in thiouracil-treated rats it may be concluded that the new compound is readily absorbed but it is not known whether it is hydrolyzed before absorption. Since starch is so readily digested it seems likely that β-amylose, a derivative of starch, should also be split to simpler units. It would be interesting to know whether ionic iodine is released in the lumen of the small intestine.

The deleterious effect of feeding tridine to growing rats is evidently closely related to an impaired appetite. The ingestion of tridine prevented maximal gains in weight. When the diet contained 5% of tridine the gain in weight was less than one-third of the control value and the food was very poorly utilized. It would be interesting to know whether this was due to non-absorption or to excessive waste after the foodstuffs had reached the blood stream.

Summary. 1. Iodinated β-amylose, tridine, prevents partially the thyroid hyperplasia caused by thiouracil. In this respect it acts like potassium iodide. Tridine, except in toxic concentrations has no effect on the metabolic rate.

2. In rats receiving thyroxin, tridine, in dosages of 1, 3 and 5% of the diet, has no significant effect on either the size of the organs or the metabolic rate.

3. In young rats all dosages of tridine (1, 3 and 5%) are deleterious with respect to consumption of food and gain in body weight.

4. In thiouracil-treated rats 3% tridine is indistinguishable in its effects from an equivalent (0.77%) amount of KI.

5. The data from the present series of experiments on 78 rats suggest that tridine when administered by mouth exerts its effect by virtue of its iodine content and when given in equivalent amounts cannot be distinguished from KI.

2 Sadhu, D. P., *Am. J. Physiol.*, 1948, **152**, 150.

16842

Lesions of the Islands of Langerhans Produced by a Styryl Quinoline Derivative.

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Dunn, Sheehan and McLetchie[1] first reported experimental necrosis of the islands of Langerhans. Although most of this report concerns the lesions produced by alloxan, they refer to some experiments in which styryl quinoline No. 90 caused injury of the islands. As the papers cited in this article contained no information concerning the effects on the pancreas, the only report of pancreatic lesions is that observed by them.[1] Since this report, Goldner and Gomori[2] have studied quinoline, cincophen and quinone. These were without effect on the pancreas. Kennedy and Lukens (unpublished) found sublethal doses of quinoline ineffective.

Because of the pancreatic damage ascribed to styryl quinoline, we have examined certain quinoline compounds with respect to their possible pancreatotoxic effect.

Methods. Normal male rabbits were kept in metabolism cages and fed an unmeasured diet of cabbage and oats. Each compound was given (a) intravenously and the response observed for 2-5 days; (b) subcutaneously every day for 2 weeks and (c) in 2 animals one compound was given by stomach tube daily for 2 weeks. The urine was tested daily for sugar, acetone, albumin and specific gravity. Occasional microscopic examinations of the urine sediments were made. The blood glucose was determined once or more on most animals. In taking the blood from the ear, the animals were not trained to this procedure, a fact which presumably accounts for the slightly elevated values obtained. Except for rabbits dying acutely from the drugs or from incidental illnesses, all of them looked well, ate well and maintained their weight. At the end of the experiments they were given nembutal and autopsied. In all cases, the pancreas, liver and kidneys were taken for microscopic examination. In a few animals other organs were sectioned also.

Two compounds were also tested in rats by subcutaneous administration.

The substances tested were as follows:

I 7-chloro-4-(4-diethylamino- 1 -methylbutylamino)- 3 -methyl*quinoline,* bis-(acid sulfate), hemihydrate.

II 7-chloro- 4 -(4-diethylamino-1-methylbutylamino)*quinoline,* diphosphate.

III 1-(7-chloro-4-quinolylamino) -3-diethylamino-2-*propanol,* diphosphate.

IV 4-(7-chloro-4-quinolylamino) -α-diethylamino-*o-cresol.*

V 2-[2-(1-amyl-2, 5-dimethyl-3-pyrryl) vinyl] - 1,6-dimethyl-quinoliniumchloride.

VI Styryl Quinoline: 2 (p-acetylaminostyryl) 6 - dimethylaminoquinolinemethochloride.

VII N-[2-(p-Diethylaminostyryl)-6-methylquinoline] ethochloride.

Results. In the case of compounds I to VI, there was neither physiological nor histological evidence of pancreatic damage. The results have been summarized in Table I. Animals dying in a few minutes have been excluded from this summary.

The kidneys deserve particular mention because of Sheehan's[1] report of nephritis from styryl quinoline. No gross injury was found in any experiments. The slight clouding on the tests for albumin never exceeded that seen in numerous normal rabbit urines. The specific gravity varied widely and included samples with good concentration, all resembling normal rabbits on the same diet.

In addition to the absence of diabetes, there was no evidence at autopsy of any harmful

1 Dunn, J. S., Sheehan, H. L., and McLetchie, N. G. B., *Lancet,* 1943, **1**, 484.

2 Goldner, M. G., and Gomori, G., *Endocrinology,* 1944, **35**, 24.

TABLE I.
Absence of Diabetogenic Effect of 6 Quinoline Compounds.

Compound (see text)	No. rabbits	Dosage		Blood glucose		Days until autopsy (range) days
		Amt mg/kg	Route*	No.	Range mg/100 ml	
I	8	10–20	i.v.	10	102-144	3–8
	2	20	oral	6	109-131	14–15
	2	20	s.c.	7	115-153	12–15
II	6	20	i.v.	6	103-121	4–5
	5	20–40	s.c.	12	102-130	15
III	6	20	i.v.	5	105-128	4–5
	4	20	s.c.	8	108-125	14
IV	6	10–20	i.v.	6	107-124	4–6
	6	20	s.c.	14	106-151	11–15
V	2	2–4	i.v.	0		1
	9 rats	4–16	s.c.	5	125-162	4–5
VI	4	10–30	s.c.	0		2

* i.v. = intravenous; s.c. = subcutaneously.

TABLE II.
Effect on Pancreatice Islets of Compound VII: N[2(*p*-Diethylaminostyryl)-6-methyl quinoline] ethochloride.

Animal and No.	No. in group	Doses × days, mg/kg	Time of autopsy, days	Necrosis of islets	Blood sugar at autopsy
		A. Lesions observed.			
Rabbit 70		15 × 1 i.v.*	<1	+++	
" 76		30 × 1 i.v.	<1	+	
Rat 1		150 × 1 s.c.	1	+	
" 3		60 × 1 s.c.	1	+	
" 5		10 × 1 s.c.	1	+	
" 6		10 × 1 s.c.	1	+	
" 10		5 × 1 s.c.	1	+	
" 17		2 × 2 s.c.	1	+	
		B. No lesions present.			
Rabbits	8	10-30 × 1 i.v.	<1 to 5	0	121-178 (5 rabbits)
Rats	12	10 × 1 to 1 × 6 s.c.	1 to 6	0	101-702 (7 rats)

* i.v. = intravenously; s.c. = subcutaneously.

effect on the liver or other organs studied. The lungs, thyroid, thymus, adrenals, spleen were all grossly normal. Microscopic examination showed that the pancreas and islands of Langerhans were entirely normal. The liver, kidney and other organs showed no definite abnormality.

Of 28 animals tested with compound VII, 8 showed evidence of early necrosis of the islands of Langerhans. Table II summarizes these experiments, and Figures 1-4 illustrate the lesions in 2 of these animals. A normal island and the early necrosis from alloxan are presented for comparison. Early necrosis is shown by the shrunken, pyknotic nuclei and by the loss of normal granulation of the cytoplasm. There was no consistent relation of these lesions to dosage; they seemed to be chance occurrences. However, lesions were seen only among the animals which died from the drug. In no instance did an animal survive with diabetes. Because of their early death, blood sugars were not obtained on the animals with lesions. Of those without lesions (series B) the blood glucose values in the rabbits are not regarded as significant. Four

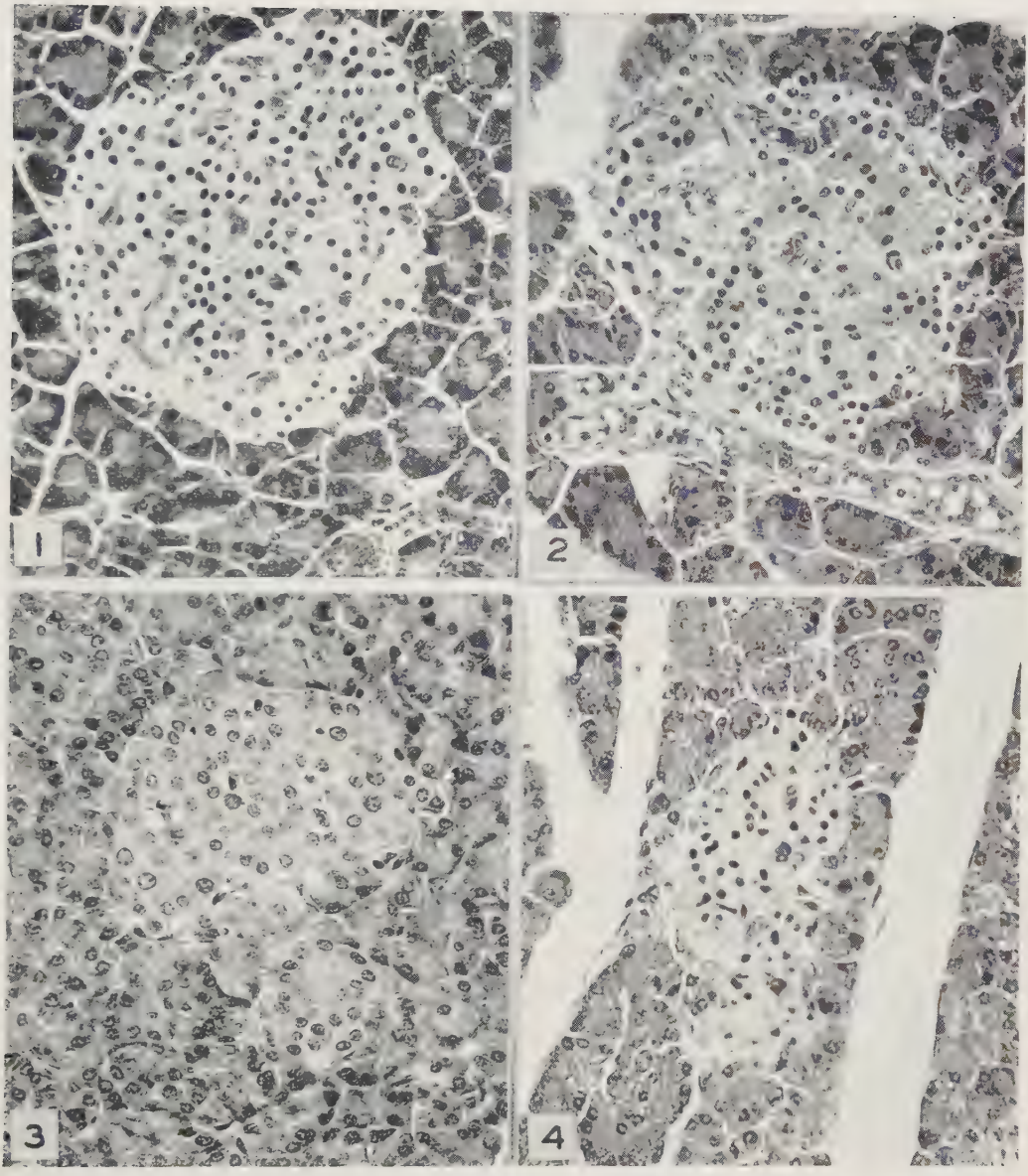

FIG. 1.
Island of Langerhans of Rat 3. Early necrosis is indicated by the pyknotic nuclei and loss of granularity of cytoplasm.

FIG. 2.
Island of Langerhans of Rat 17 showing early necrosis.

FIG. 3.
Normal island (rabbit).

FIG. 4.
Necrosis of island 11 hours after alloxan (rabbit).

of the rats without lesions had very high blood sugars indicative of diabetes but these animals were moribund at the time the blood sugars were determined. In all of 5 rabbits and 7 rats in which the kidney was examined microscopically, there was tubular necrosis of varying severity.

Discussion. The statement that adminis-

tration of compounds I to VI caused no pancreatic damage requires the following comment:

(a) The blood sugars were frequently above the normal level and this has been attributed to the technique used. When normal rabbits were handled in this manner blood sugars have been of this magnitude. There is a clear distinction between these values and those of rabbits with alloxan diabetes in which the blood sugars are characteristically high *i.e.*, 300 to 500 mg per 100 cc. The consistently negative urines provided a mass of evidence, not included in the tables, that these rabbits were not diabetic as a result of the quinoline compounds.

(b) The conclusion that these compounds are not diabetogenic is based on results in a single species. Although the rabbit was used by Sheehan[1] in the study of styryl quinoline, and although the rabbit is the animal most susceptible to alloxan, this limitation must be mentioned.

(c) The tendency to compare other toxic chemicals with alloxan may lead to errors in spite of every effort that has been made to observe diabetogenic activity. Thus, alloxan in similar sublethal doses would have caused diabetes with certainty, within the time limits of these experiments. A more insidious toxin could theoretically escape detection.

As far as we know, the results obtained with compound VII provide the first confirmation of Sheehan's[1] report that styryl quinoline caused injury of the pancreatic islands. As we gave styryl quinoline to only 4 animals our negative results with this compound (VI) might be accidental. This series was small because of Bailey's personal communication that he had not produced lesions of the islands or diabetes with styryl quinoline. The presence of lesions in the larger series of animals given a derivative of styryl quinoline (VII) is in general accord with Sheehan's[1] observations. He described "from memory" the consistent occurrence of islet necrosis after lethal doses of styryl quinoline (VI). In our series lesions occurred in the islets only after lethal doses but their occurrence was quite inconstant. In view of the absence of detailed protocols in the original report[1] further discussion is omitted.

Summary. Six quinoline derivatives have been administered intravenously and subcutaneously to rabbits and rats. There was no functional or anatomical evidence of damage to the islands of Langerhans or to any other organ under the conditions employed.

A seventh compound, N- 2-(p-Diethylaminostyryl)-6-methyl quinoline-ethochloride caused lesions of the islands resembling the early necrosis produced by alloxan. The lesions occurred in 8 of 28 animals tested. Islet necrosis was always associated with lethal dosage but death from the drug was not always accompanied by necrosis. Tubular damage in the kidney was found in all cases examined. Survival with diabetes was not observed.

16843

Potential Antigenicity of Parenterally Administered Cytochrome C.

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The separation and purification of cytochrome C by Keilin *et al.*[1] opened the field for its use in animal and human experimentation, in which encouraging results have since been claimed.[2-4] Its production usually involves

[1] Keilin, D., Hartree, E. F., and Hartree, F. R. S., *Proc. Roy. Soc. Med.*, 1937, **122**, 299.

[2] Proger, S., *Bull. New England Med. Center*, 1945, **7**, 1.

[3] Proger, S., and Decaneas, D. J., *Bull. New England Med. Center*, 1945, **7**, 149.

[4] Frank, H. A., Seligman, A. M., and Fine, J., *J. Clin. Invest.*, 1945, **24**, 435.

extraction from horse or beef heart tissue, but the possibility of antigenicity has not been noted in any of the papers on cytochrome C seen to date, and only one brochure with a commercially available preparation suggests such a possibility.

Experimental. Three preparations of cytochrome C of different manufacturers were obtained; two were of equine origin, and one was of mixed bovine and equine origin. (For convenience, these preparations hereafter will be referred to respectively as CC-I, CC-II, and CC-III). All sensitizing doses were intraperitoneal. All shocking doses were via the dorsal penile vein.

Fourteen male guinea pigs were given 3 successive sensitizing injections of CC-I intraperitoneally at 2-3 day intervals. Group I, (9 animals), received 1 mg per dose, and Group II, (5 animals), received 5 mg per dose. In Group I, 3 weeks after the last sensitizing injection, 6 were challenged by the injection of CC-I via the dorsal penile vein. There was one fatal anaphylactic shock, 4 reactions with recovery, and one negative result. One week later the 3 of this group not previously tested were similarly challenged, and all died of anaphylactic shock. In Group II, all 3 animals challenged at 3 weeks died of anaphylactic shock, as did also the remaining 2 challenged at 4 weeks.

In a second experimental series, giving only 2 sensitizing injections of 2 mg per dose, 6 animals were sensitized to CC-I and 6 received similar doses of CC-II. Nineteen days later each group was divided so that 3 received the original sensitizing material, and the other 3 received the alternate product. None of the animals sensitized to CC-I showed any reactions, either to CC-I or CC-II. On the other hand, all animals initially sensitized to CC-II exhibited some degree of anaphylaxis when challenged with either CC-I or CC-II, and one death occurred with each material.

A third series of 9 guinea pigs was sensitized with CC-III, in 2 successive doses of 2.5 mg each. One month later 4 were challenged with the same material, 3 mg intravenously. None showed any shock symptoms. The remaining animals were challenged 5½ weeks after the last sensitizing dose, and none of these showed any reactions. Those tested at one month were again challenged 10 days later, and again no reactions were observed.

That the reactions observed in the first two series are due to primary antigenicity and not to a possible horse serum contamination was shown in a control series of 7 guinea pigs sensitized to horse serum. One animal given 0.75 cc horse serum died of anaphylactic shock. The 6 others received CC-I in doses from 3 to 10 mg and none showed any symptoms of shock. Exhaustion of the limited supply of the same lots of cytochrome C made it impossible to pursue further reciprocal testing with horse serum.

Discussion. The above experiments show that the clinical application of cytochrome C may be attended by a hazard similar to that experienced with horse serum. Attention should be drawn to some of the variations noted in the above experiments. Anaphylactic symptoms were more frequent and severe when the sensitizing doses were multiple and large, and when the incubation period was prolonged. When the number or quantity of sensitizing doses was reduced, anaphylactic symptoms diminished or were absent, so that no antigenicity was demonstrated under the experimental conditions. However, we have shown[5] that weakly antigenic materials, given a greater number of times for sensitization, and allowed a longer incubation period, may be shown to possess antigenicity not demonstrable by more commonly employed methods.

The possibility of a serious or even fatal anaphylactic reaction in the potentially sensitive human patient must therefore be considered when cytochrome C is administered intravenously, particularly if more than once, and testing precautions should be taken.

Summary. Guinea pigs sensitized with cytochrome C of equine origin when challenged with the same material, were shown to exhibit anaphylactic reactions, ranging to fatal, in a relatively large percentage of animals. Cross sensitivity could not be demonstrated between cytochrome C and horse

[5] Roth, L. W., Richards, R. K., and Shepperd, I. M., *Fed. Proc.*, 1948, **7**, 105.

serum, but remains a possibility. Cross sensitivity to cytochrome C of different manufacture was demonstrated. The possible hazards of its use clinically are emphasized.

16844

Assay of Anti-Pernicious Anemia Factor with *Euglena.*

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It was noted that massive growth of the algal flagellate *Euglena gracilis* depended on unknown growth factors present in crude casein but absent in certain plant proteins such as edestin and concanavallin A.[1] This factor was removed from casein by repeated isoelectric precipitation. It was subsequently found that alcoholic extracts of crude casein‡ were active, and refined liver extract was recently reported to possess considerable activity.[2] It was found in the present investigation that this growth factor requirement was satisfied by a combination of crystalline anti-pernicious anemia factor (APA)[3] plus thiamine. These findings became the basis of an assay method for APA.

Trial of APA was suggested by the good agreement between the animal protein factor (APF) activity as measured with chicks, and the growth-promoting effect for *Euglena* of injectable liver extracts and of microbial APF concentrates. The latter were found to produce an hematopoietic response in pernicious anemia.[4]

Experimental. The organism used was *Euglena gracilis* var. *bacillaris.* The culture vessels were exposed to "daylight" fluorescent lamps at 25° to 31°C. The optimal temperature was 28° to 31°C; inhibition effects began to appear at approximately 32°C. At first, assays were carried out in 25-ml Erlenmeyer flasks covered with glass caps, and containing 10 ml of medium. It was later found convenient in routine assays to employ 100 x 13 mm tubes containing 2 ml of medium, illuminated from below. The light was supplied by 4, 40-watt lamps mounted side by side, 30 cm from the cultures. Light intensity did not appear to be a limiting factor at the levels of growth reported here. The basal medium is shown in Table I. The thiamine requirement was approximately 0.5 mμg/ml for half

TABLE I.
Composition of Basal Medium.

	Per l final medium (pH 6.5)
$NH_4H_2PO_4$	0.8 g
Potassium citrate, monohydrate	0.2 "
$MgSO_4 \cdot 7H_2O$	0.2 "
Sodium butyrate	2.0 "
Monosodium glutamate	1.0 "
$CaCl_2$	0.1 "
$FeSO_4 \cdot 7H_2O$	20 mg
$MnSO_4 \cdot H_2O$	6 "
$CoSO_4 \cdot 7H_2O$	5 "
$ZnCl_2$	0.8 "
$Na_2MoO_4 \cdot 2H_2O$	1.0 "
$CuSO_4 \cdot 5H_2O$	0.08 "
Thiamine chloride	0.1 "

* Haskins Laboratories. Aided by a grant from Lederle Laboratories Division, American Cyanamid Co.

† Lederle Laboratories Division, American Cyanamid Co.

1 Hutner, S. H., *Arch. Protistenk.*, 1936, **88**, 93.

‡ Generously supplied by the Nutritional Biochemicals Corporation, Cleveland, Ohio.

2 Provasoli, L., Hutner, S. H., and Schatz, A., PROC. SOC. EXP. BIOL. AND MED., in press.

3 Smith, E. L., *Nature*, 1948, **161**, 638. A sample was kindly furnished by the Glaxo Laboratories, Ltd.

4 Stokstad, E. L. R., Page, A., Pierce, J., Franklin, A. L., Jukes, T. H., Heinle, R. W., Epstein, M., and Welch, A. D., *J. Lab. Clin. Med.*, 1948, **33**, 860.

TABLE II.
Growth of Euglena in the Basal Purified Culture Medium with Various Supplements.

Basal medium used	APA factor added per ml of medium (mμg)	Other additions per ml of medium	Incubation period (hr)	Growth (optical density)
A	None	None	115	.04
,,	.0015	,,	,,	.18
,,	.005	,,	,,	.42
,,	.015	,,	,,	.92
,,	.05	,,	,,	1.34
,,	.15	,,	,,	1.42
,,	.5	,,	,,	1.40
,,	None	0.05 mμl liver extr.	,,	.14
,,	,,	0.15 ,, ,, ,,	,,	.42
,,	,,	1.0 ,, ,, ,,	,,	1.08
,,	,,	3.0 ,, ,, ,,	,,	1.20
,,	,,	10 ,, ,, ,,	,,	1.40
B	None	None	102	.07
,,	.15	,,	,,	.21
,,	,,	0.05 mμg thiamine HCl	,,	.34
,,	,,	0.15 ,, ,, ,,	,,	.52
,,	,,	0.5 ,, ,, ,,	,,	.97
,,	,,	1.5 ,, ,, ,,	,,	1.32
,,	,,	5 ,, ,, ,,	,,	1.30
,,	.5	Vitamin mixture* without thiamine	92	.20
,,	1.0	.05 μg nicotinic acid	,,	.36
,,	,,	0.5 μg pantothenic acid	,,	.35
,,	,,	5 μg pyridoxine HCl	,,	.28
,,	,,	0.05 μg biotin	,,	.30
,,	,,	0.5 μg pteroylglutamic acid	,,	.24
,,	,,	5 μg *p*-aminobenzoic acid	,,	.32
,,	None	None	120	.13
,,	1.0	0.5 μg thiamine HCl	,,	1.40
,,	None	0.5 μg thiamine HCl plus 0.5 μg thymidine	,,	.12
,,	,,	0.5 μg thiamine HCl plus 1.5 μg thymidine	,,	.11
,,	,,	0.5 μg thiamine HCl plus 5 μg thymidine	,,	.12

A—Basal medium as in Table I.
B—Basal medium as in Table I except that thiamine was omitted.
* Riboflavin, niacin, pantothenic acid, pyridoxine, 0.5 μg each, biotin 0.05 μg.

maximum growth and 2.0 mμg/ml for maximum growth. Growth was practically complete in tubes in 4 days when a heavy inoculum was used. The inoculum was prepared by growing the organisms in 50-ml flasks containing 10 ml of basal medium supplemented with sufficient refined liver extract to allow about two-thirds maximum growth; one drop of a dense vigorous culture was then added to each tube or flask. Stock nutrient solutions were preserved by adding a mixture of *o*-fluorotoluene, 1, 2-dichloroethane, and *n*-butyl chloride.[5]

Results and Discussion. The results of some typical experiments are shown in Table II. It was found that about 0.01 mμg of APA factor per ml was required by *Euglena* for "half-maximum growth". This level is only approximately one-tenth as great as that required by *Lactobacillus leichmannii* 313.[6] Thymidine[§] was inactive when tested up to

[5] Hutner, S. H., and Bjerknes, C. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 393.

[6] Hoffmann, C. E., Stokstad, E. L. R., Franklin, A. L., and Jukes, T. H., *J. Biol. Chem.*, 1948, **176**, 1465.

[§] We are indebted to Dr. D. W. Woolley for a sample of thymidine from the Levene collection, to Dr. J. O. Lampen for a sample prepared by him by the method of Klein,[7] m.p. 184.5° to 185.5° (uncorrected), and to Dr. W. Shive for a third sample.

[7] Klein, W., *Z. physiol. Chem.*, 1948, **255**, 82.

10 μg/ml. The lack of response to thymidine, in contrast to the effectiveness of thymidine for lactobacilli[8–10] draws attention to the value of comparative studies of *Euglena* and lactobacilli in exploring the functions of APA. Biochemical generalizations regarding these functions in various species should probably not be made solely on the basis of the observations with lactobacilli.

Experiments now in progress indicate that this preliminary assay medium is capable of further improvements.

Summary. The algal flagellate *Euglena gracilis* var. *bacillaris* was shown to exhibit a quantitative growth response to crystalline antipernicious anemia factor, using a chemically defined medium. Thymidine was inactive.

8 Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Chem. Soc.*, 1948, **70**, 2614.

9 Snell, E. E., Kitay, E., and McNutt, W. S., *J. Biol. Chem.*, 1948, **175**, 473.

10 Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, 1948, **175**, 475.

16845

Varying Effect of Thyroxine on Oxygen Consumption of Different Tissues.

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Some tissues from hyperthyroid animals preserve their increased metabolism after isolation from the animal. Tachycardia and increased oxygen consumption of the heart persist for hours.[1,2] Similarly, striated muscle and kidney tissue show a greatly increased oxygen consumption.[3] This shows that the increased metabolism is due to biochemical alterations in the cell, and not to nervous influences. It was thought by us that thyroxine might affect all actively metabolizing tissues in the same way. The experiments here reported show that this does not hold for brain cortex slices, which preserve a normal oxygen uptake despite marked hyperthyroidism in the intact animal. Liver, on the other hand, shows a peculiarly variable response.

Adult male albino rats (Donaldson strain) were used in all experiments. Thyroxine was injected subcutaneously in doses from 2.5 to 10 mg per kg every other day. The animals were sacrificed at from 7 to 21 days. Qo_2 of slices of gray matter was determined by the Warburg method, in 100% O_2, using phosphate buffer and glucose as substrate. In 26 experiments the Qo_2 of brain was found to be between 9.0 and 13.0 in all but 4 instances. In these 4 instances there was moderate increase ranging from 14.9 to 18.7. The mean Qo_2 for hyperthyroid brain was 11.5, which is exactly the same as that found in 13 experiments with normal brain (Table I).

In 14 experiments the Qo_2 of kidney slices from the same animals was determined. It was found to be greatly increased, averaging 36.1, or almost double the normal value. In 6 experiments the Qo_2 of small sheets of diaphragm muscle averaged 8.1, an increase of about 70% over the normal. The high rate of respiration of muscle and kidney tissue confirmed observations made by one of us some years earlier.[3]

In 13 instances the B.M.R. of the animal was followed, up to the time of sacrifice. This was done by the method of Tainter and Rytand.[4] All the animals were markedly hyper-

1 Lewis, J. K., and McEachern, D., PROC. SOC. EXP. BIOL. AND MED., 1931, **28**, 504.

2 McEachern, D., *Bull. Johns Hopkins Hosp.*, 1932, **50**, 287.

3 McEachern, D., *Bull. Johns Hopkins Hosp.*, 1935, **56**, 145.

4 Tainter, M. L., and Rytand, D. A., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 361.

TABLE I.
Oxygen Consumption of Isolated Tissues from Normal and Hyperthyroid Animals.

	Q_{O_2} tissue (cu mm O_2/mg dry wt/hr)			B.M.R. animal (cc O_2/g/hr)		
	Brain	Diaphragm	Kidney	Initial	Final	Increase
Hyperthyroid series						
Mean	11.5	8.1	36.1	1.69	3.65	+116%
No. of exper.	(26)	(6)	(14)	(13)	(13)	(13)
Normal series						
Mean	11.5	4.8	22.5			
No. of exper.	(13)	(23)	(10)			
T.*	0	8.1	4.5			

* T. is the ratio of the difference to the probable error of the difference. It is only considered to be significant when the ratio is 3:1 or greater.

thyroid, as evidenced by the fact that their basal metabolic rates showed increases of between 84 and 201%. The mean increase for the group was +116%.

It is possible that brain metabolism may be somewhat increased during the first few days of thyroxine administration, and that later an anti-hormone effect may come into play. Of the 4 instances in which we obtained increases of oxygen consumption, 3 occurred in animals given thyroxine for only 7 to 9 days, whereas the majority received it for about 15 days. MacLeod and Reiss[5] found that after treatment of hypophysectomized rats with thyrotrophic hormone there was a moderate increase of oxygen consumption of brain slices between 5 and 8 days after commencement of treatment. After 16 days treatment the effect on brain was no longer apparent. Jandorf and Williams[6] found a reversion to normal in the B.M.R. of rats and the oxygen consumption of their isolated tissues after about 3 weeks treatment with thyrotrophic hormone. Certainly with thyroxine the increased oxygen consumption of muscle and kidney tissue, as well as that of the intact animal, is maintained over the period. This is not so for brain, and it may be an interesting example of homeostasis for a very select organ.

It is not clear why brain tissue fails to show the increase of oxygen consumption so characteristic of other tissues. It fits, however, with the observation of Gross and Leblond[7] who found that both white and gray matter showed minimal take-up of thyroxine labelled with radioiodine.

TABLE II.
Effect of Succinate on Oxygen Consumption of Liver Slices.

Hyperthyroid Q_{O_2}	Mean Q_{O_2} of liver		% Increase
	Glucose	Succinate	
0– 5	3.6	26.1	+625
5–10	7.7	53.2	+590
10–20	13.2	60.4	+357
Normal	11.2	39.4	+251

A curious phenomenon was found with hyperthyroid liver. The Q_{O_2} of normal liver is about 10.0, and liver from some hyperthyroid animals showed increases up to twice normal. Liver from other animals, however, gave Q_{O_2}'s that were practically nil. As shown in Table II, the Q_{O_2} was below 10.0 in 14 instances, in one case being as low as 1.3. In 13 experiments the Q_{O_2} was above 10.0, in one case reaching 21.3. This marked variability was at first attributed to some technical error but it soon became clear that this was not so. The phenomenon of very low Q_{O_2} was only found in the hyperthyroid series, and not with the normal controls which were run at the same time and which always gave Q_{O_2}'s ranging from 9.0 to 12.0. Duplicate runs were made on hyperthyroid liver and

[5] MacLeod, L. D., and Reiss, M., *Biochem. J.*, 1940, **34**, 820.

[6] Jandorf, B. J., and Williams, R. H., *Am. J. Physiol.*, 1944, **141**, 91.

[7] Gross, J., and Leblond, C. P., *J. Biol. Chem.*, 1947, **171**, 309.

they agreed satisfactorily. Furthermore, the very low rates of respiration were only found with hyperthyroid liver and not with other tissues. The phenomenon may be due to liver damage or to the exhaustion of some substrate or enzyme system within the cells.

When M/48 succinate was added at the end of the first hour it was found that the low-respiring liver slices were stimulated to remarkable activity, as shown in Table II. Here the results on hyperthyroid liver are considered in 3 groups, depending on the original Q_{O_2} of the tissue. The mean per cent increase for the lowest respiring group was 625%, considerably greater than for those tissues having a higher initial respiration. Suitably thin slices of tissue were chosen to meet the high O_2 consumption.[8] Increases of respiration were not as great as those obtained by the above author with starved liver since we used M/48 succinate instead of the M/25 optimal concentration. The differential increase between normal and hyperthyroid liver slices is clear, however. Our results show that the low-respiring liver slices may have difficulty in using glucose as substrate but are capable of using succinate.

Numerous experiments were carried out to try to pin down the cause of the low respiration in liver of some hyperthyroid animals, but without success. Three different types of diets were tried, one heavily supplemented with vitamins and minerals. These were Fox Chow, the diet of Sherman and Sandels[9] and that of McEachern.[3] The method of killing the animal was explored, using ether, gas and drowning in different experiments. The effect of fasting or of feeding right up to the time of sacrifice was investigated. None of the above factors appeared to play a role.

Rosenthal[8] found that the intensity of spontaneous respiration and the oxidation of lactate and pyruvate by liver slices is decreased in starved animals, but that the intensity of succinate oxidation is not influenced by starvation. He suggested that a decrease in the concentration of co-enzymes in the cell may be responsible since the oxidations of lactate and pyruvate, in contrast to the oxidation of succinate, are known to require co-enzymes. Peters and Rossiter[10] showed that rapid depletion of co-carboxylase occurs in the livers and other tissues of hyperthyroid rats fed upon a basal diet and that this defect is somewhat repaired by administration of thiamin. It is possible that hyperthyroidism may deplete the liver in some such way even when the animals are offered an adequate diet.

Summary. Cerebral gray matter of hyperthyroid rats does not show the increase of respiration shown by the intact animal or by diaphragm muscle or kidney tissue. Liver tissue shows a highly variable respiration in hyperthyroidism, sometimes being abnormally low. These low-respiring tissues are, however, greatly stimulated by the addition of succinate. It would seem that in hyperthyroid liver there may be a shortage of co-enzyme for the glucose-pyruvate cycle. The cytochrome-oxidase system, as judged by succinate response, remains intact or is enhanced.

8 Rosenthal, O., *Biochem. J.*, 1937, **31**, 1710.

9 Sherman, H. C., and Sandels, M. R., *J. Nutrition*, 1931, **3**, 395.

10 Peters, R. A., and Rossiter, R. J., *Biochem. J.*, 1939, **33**, 1140.

16846

Effect of Vagus on the Monophasic Action Potential of Auricular Muscle.

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A turtle auricular strip is deeply immersed in a large volume of Ringer's solution. Arrangements are made for unipolar recording of the action potentials. Non-polarizable electrodes are used in conjunction with a Cambridge All-Electric Electrocardiograph. A drop of mecholyl (acetyl-beta-methylcholine chloride; $1:10^4$) is placed on the tissue near the tip of the recording electrode. A stimulus is now applied to one end of the strip and a triphasic curve is recorded which looks like the membrane current curve of nerve. If one performs two successive integrations on this curve, or uses the graphical method recently described (Churney, Ashman, and Byer[1]), the monophasic action potential curve may be obtained. This monophasic curve resembles that of nerve and differs from that of the uninhibited auricle in at least two respects: (1) the descending limb characterizing the repolarization process is convex towards the time axis rather than concave; and, (2) its duration is shorter. By means of the suction electrode[2] one can experimentally verify this theoretically derived monophasic curve.

Another method for studying these phenomena, and one which yields additional information, is as follows. A cotton wick electrode soaked in isosmotic potassium chloride solution is fixed to the apex of the left auricle. The other electrode is placed either on the surface of the left auricle, or in the body cavity. The left vagus is exposed in the neck and prepared for stimulation.

Recording with the KCl-treated electrode on the auricle in conjunction with a remote electrode lends itself, we believe, to a very simple interpretation. Almost surely we are recording the potential changes of a ring of injured cells whose inner surfaces are in electrical communication with the KCl-treated lead. We find a ready interpretation of our results by treating the situation as though it were a single spherical cell, one side of which has its charges partially or entirely wiped out. On, or close by, this latter surface is the recording electrode; the other being remote.

The result of vagal stimulation on the auricular monophasic curve is shown in Fig. 1. In addition to showing clearly the effects mentioned already, the "spike" voltage is seen to be greatly reduced. (The quantitative aspects of the fall and recovery in the magnitude of the spike are of no consequence for the moment). So, even though there is only a very slight increase in the threshold of excitation (Ashman and Garrey[3]), provided the inhibition is partial, the action currents originating in the sinus give rise to potentials in the auricle which are below normal. This record, as well as others obtained from linear strips treated with mecholyl, shows that the monophasic action potential during vagal stimulation is not associated with a change in the level of polarization of the resting membranes. We have never observed a Gaskell effect.

The question now confronting us is this. Is the decrease in spike voltage real, or only apparent? More specifically, is it associated with a partial depolarization of the individual auricular muscle fiber, or does it result from the irresponsiveness of various fibers? Early investigators (Gaskell,[4] Rossbach,[5] and oth-

* Address: College of Medical Evangelists, Loma Linda, Calif.

1 Churney, L., Ashman, R., and Byer, E., *Am. J. Physiol.*, 1948, **154**, 241.

2 Wiggers, H. C., *Am. J. Physiol.*, 1937, **118**, 333.

3 Ashman, R., and Garrey, W. E., *Am. J. Physiol.*, 1931, **98**, 109.

4 Gaskell, W. H., *J. Physiol.*, 1880, **3**, 48.

5 Rossbach, M. J., *Arch. f. ges. Physiol.*, 1882, **27**, 197.

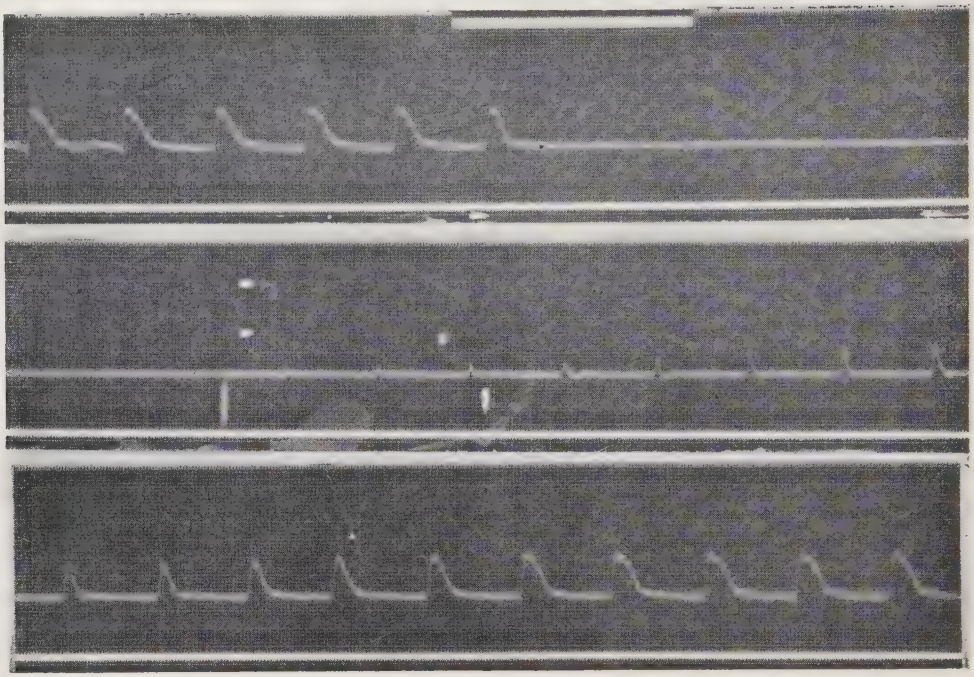

FIG. 1.

Effect of vagal stimulation on the monophasic action potential records of turtle auricular muscle. One electrode is on the KCl-treated apex of the left auricle, the other on the uninjured surface. The ventricle has been tied off to prevent its beating. Upper signal: left vagus stimulated faradically. Calibration: 3 millivolts.

ers) definitely assumed that the negative inotropic effect is the result of the diminished force of contraction of the entire syncytium, but without any real proof. See, too, the review of Adrian.[6] Concerning the reality of the modified spike potential itself there can be no doubt, since the theoretically predicted triphasic volume conductor curve is experimentally recorded.

Now there is no doubt that under some conditions mecholyl can cause blocking, and so the decrease in spike magnitude could be apparent only. However, at least four lines of evidence support the alternative viewpoint and are incompatible with the blocking concept. First, we recall the observation that there is a drastic change in the *form* of the monophasic action potential curve. Furthermore, there appears to be a strict association between the spike height and the shape of the recovery limb (Fig. 1). Not only is the convexity (towards the time axis) the more pronounced the less the apparent degree of depolarization, but the convexity appears *pari passu* with the decrease in spike magnitude following vagal stimulation, and does not become reversed during recovery until the spike is of full height, or very nearly so (Fig. 1). Secondly, we have confirmed the well known observation that vagal stimulation shortens the absolute refractory period of partially inhibited auricular muscle (Gilson[7]). There is some evidence that the less the spike height, or the shorter the duration of the electrical complex, the greater the percentage shortening of the absolute refractory period. With a decrease in spike amplitude to 17.9% of the original value, the "absolute refractory period" (as measured from the beginning of the spike to the signal of the applied stimulus) fell to at most 35.4% of its value for the unatropinized muscle. According to Asher and Scheinfinkel,[8] acetylcholine also shortens the absolute refractory period of nerve.

[6] Adrian, E. D., *Ergeb. d. Physiol.*, 1933, **35**, 744.

[7] Gilson, A. S., Jr., *Am. J. Physiol.*, 1935, **112**, 610.

Thirdly, we cite the finding of Gilson[9] that there is a very great increase in accommodation of turtle auricular muscle following vagal stimulation. If one defines accommodation in the most general terms: namely, as an active resistance of the tissue to depolarization, then this phenomenon may be the excitation counterpart of the decrease in spike voltage and accelerated repolarization seen in the electrograms.

Finally, insofar as the duration of the monophasic action potential curve and the absolute refractory period are measures of the length of the depolarized zone,[10] we should expect this length to be considerably reduced by vagal stimulation. Some idea of the order of magnitude of this reduction may be calculated as follows. From diphasic records of linear strips in air the speed of conduction may be taken, roughly, as 0.5 m./sec., though this may be a slight underestimate. At room temperature (30°C) the duration of the monophasic curve of the uninhibited muscle is about 0.8 sec. The depolarized area, by far the greater part of which is in the state of repolarization, is therefore 40 cm long. The duration of the shortest monophasic curve following vagal stimulation is 0.04 sec. (Fig. 1), and, since there is no apparent change in conduction speed, its length is 2 cm.

Now, if the decrease in spike amplitude is real, the degree of depolarization of the inhibited muscle, as well as its length, must be very much less than that of normal, uninhibited muscle. If the extent of depolarization is not different, then, leading from the injured to the uninjured surface, a decrease in the diameter of the electrode on the uninjured surface should give a considerable increase in the percentage amplitude of the spike of the inhibited muscle. The fact that no such increase was found may be taken as further evidence that the decrease in spike height following vagal stimulation is associated with a partial depolarization of the individual heart muscle fiber.

Summary. The effects of vagal stimulation on the form of the monophasic action potential of auricular muscle are listed along with the technics for demonstrating them. Evidence is presented for the viewpoint that auricular muscle, following vagal stimulation, acts like a single cell capable of giving graded electrical, as well as mechanical, responses.

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9 Gilson, A. S., Jr., *Am. J. Physiol.*, 1939, **127**, 333.

10 Lloyd, D. P. C., in Fulton's revision of Howell's Textbook of Physiology, Philadelphia, 1941.

16847

Failure of Rutin to Decrease the Mortality of Acute Ionizing Radiation Illness in Mice.*

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Rutin, a crystalline rhamnoglucoside of quercetin, has been reported to influence favorably the hemorrhagic syndrome of acute ionizing radiation illness in dogs.[1] It has been found to accelerate the healing of the skin lesions of rats induced by localized radiation injury.[2] It seemed desirable, therefore, to study the effect of rutin on the mortality of

* Project NM 007 039, Report No. 16, Naval Medical Research Institute, Bethesda, Md., August 19, 1948.

† The opinions or assertions contained herein are the private ones of the authors and are not to be considered as official or reflecting the views of the Navy Department or the naval service at large.

1 Rekers, P. E., and Field, J. B., *Science*, 1948, **107**, 16.

acute irradiation illness in mice.

Materials and methods. White, Swiss, female mice, obtained from this Institute's colony 30 days after weaning, were fed ground Purina mouse pellets to which had been added 35 mg of ascorbic acid and 16 g of Brewer's yeast per 1000 g of food. They were approximately 7 to 8 weeks old when irradiated, having been isolated and observed for a period of at least 10 days before use in any experiment.

Rutin administration was commenced either 2 weeks prior to, or on the day of, irradiation. In one group receiving rutin *before irradiation,* the drug was given in the following doses: One hundred were given a saturated solution of rutin in water to drink (13 mg/100 cc). Ninety-nine were given a solution of 10 mg/100 cc in drinking water. One hundred received the saturated solution of rutin in the drinking water plus 20 mg of rutin per 100 g of ground food. In another group 99 animals received rutin beginning *on the day of exposure* (saturated solution in water plus 20 mg/100 g of food). All animals received the drug continuously for the 28 day post exposure observation period. The 196 control animals for the above groups received the same amount of radiation.

All irradiated control and treated mice of a given group were exposed simultaneously. The source of radiation was the angular beam of a 1000 KV, 3 ma, G.E. Industrial X-ray machine, the characteristics of which have been previously described.[3] The animals were exposed in specially designed cages constructed of 3/16″ plywood. Each cage consisted of two tiers, each holding 25 mice, and conformed in shape to a segment of a circle with a one-meter radius. The tops and bottoms of the cages were made of window screening for ventilation. Details of the construction of the cages and of their placement are reported elsewhere.[4] Animals from each group were marked and uniformly distributed in the cages around the tube so that each segment of the circle contained a representative number from each group. By this method it was possible to irradiate 400 mice simultaneously.

The average output of the tube was 31.2 r/min. at the geometric center of the cages, which was 104.4 cm from the center of the target. The variation in irradiation output as measured at the center, front, back, top and bottom of each cage was not more than ±0.5 r/min. The exposure period was 22.5 minutes for all mice (a calculated dose of 702 r plus an estimated 3 r from the warm up of the tube, or a calculated total dose of 705 r ± 11 r.

All mice were weighed daily before and after the exposure to radiation. Cages were inspected in the morning and the evening for dead animals. A few gross pathologic examinations were performed. No microscopic examinations were made. In general the study consisted exclusively of survival and weight recordings.

Results. The administration of rutin alone to mice resulted in no evidence of toxicity. The usual clinical signs of radiation illness in both the control and rutin-treated *groups of irradiated* animals were identical. By the end of the 28-day observation period all surviving animals appeared well and were gaining weight.

In the first experiment there were 96 control mice and 99 experimental mice that received rutin for 2 weeks before irradiation. Mice began to die in the control group on the ninth day after exposure and in the rutin group on the eighth day. After the nineteenth day there were only scattered deaths. From this initial experiment it appeared that the mortality was increased from 40.6% in the control animals to 52.5% in the rutin-treated animals. The probability of these data being from the same distribution is between 0.10 and 0.20 as determined by the Chi

[2] Griffith, J. Q., Jr., Anthony, E., Pendergrass, E. P., and Perryman, R., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 332.

[3] Tullis, J. L., Tessmer, C. F., Cronkite, E. P., and Chambers, F. W., Jr., Naval Medical Research Institute, Project NM 007-039, Report No. 3, Dec. 1947, in press, *Radiology,* 1949.

[4] Chapman, W. H., Sipe, C. R., Eltzholtz, D. C., Cronkite, E. P., Lawrence, G. H., and Chambers, F. W., Jr., Naval Medical Research Institute, Project NM 007-039, Report No. 14, July 1948.

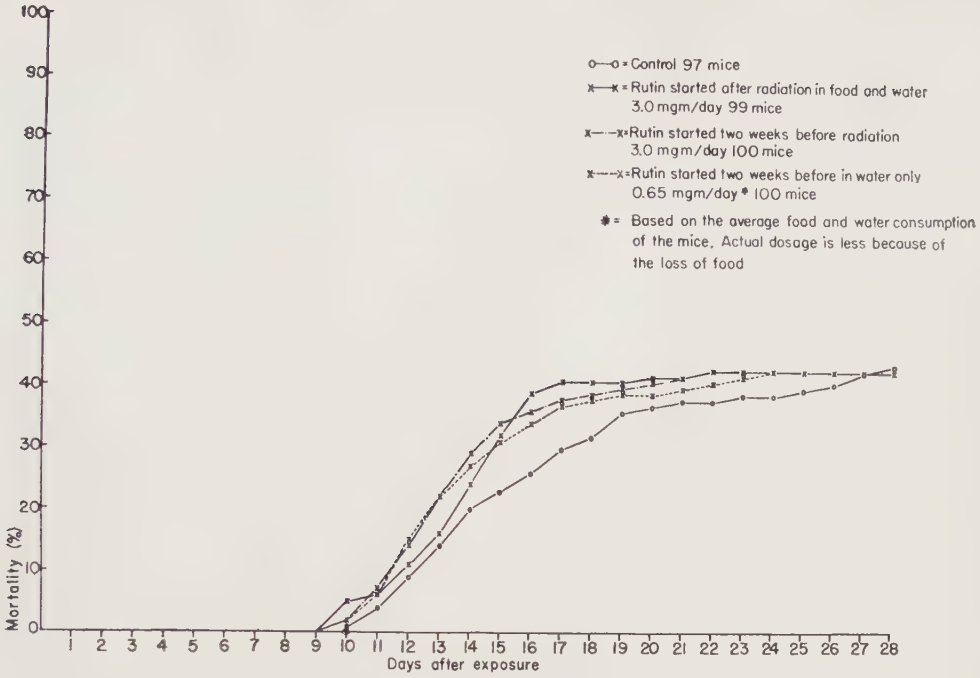

Figure 1.— Mortality curves of the control mice and the three rutin groups

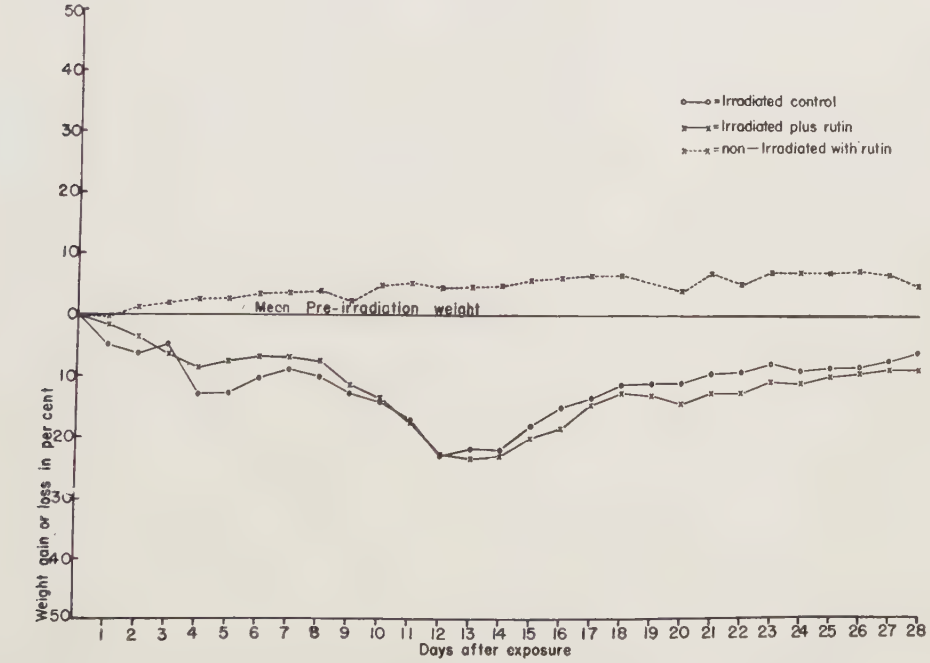

Figure 2.— Pooled average weight curves of mice in terms of percent loss or gain over the pre-experimental weight.

square method. Hence, the difference in results is not significant.

Fig. 1 shows that dosage and the time of administration did not significantly affect the results. The ultimate pooled mortality of the treated and non-treated mice on the 28th day was not significantly different ($0.70 > P_{diff} > 0.50$); however, on the 17th day the mortality of the rutin-treated groups was significantly greater than that of the non-treated

groups ($0.05 > P_{diff} > 0.02$). This is further emphasized by the shorter average survival time of the combined rutin-treated animals that died (14.1 days) as compared to 15.4 days for the non-treated.

In Fig. 2 the pooled weights of all irradiated control mice, rutin-treated mice, and rutin-treated non-irradiated mice are plotted in terms of per cent gain or loss of original pre-irradiation weight. There was no significant difference between the irradiated control and irradiated rutin-treated mice. Maximum total weight loss was obtained by the 12th day after exposure when the mortality rate was at a maximum. The weight loss and mortality decreased simultaneously. However, scattered deaths did occur after the average weight began to increase. At the end of the observation period the average weight was still below that of the pre-irradiation period. The rutin, non-irradiated mice maintained a normal growth rate.

Autopsies of selected animals demonstrated the usual picture of irradiation illness in mice that has been adequately described in the past.[5]

Comment. The original attempt of Rekers and Field[1] to control the hemorrhagic syndrome of irradiation illness was based on the favorable clinical reports of rutin in the management of increased capillary fragility.[6,7] The results reported by Rekers and Field on the use of rutin in irradiated dogs were most encouraging. However, the *modus operandi* of rutin in controlling capillary fragility in general or in producing the favorable results of Rekers and Field in dogs is not known. It has been emphasized that rutin is less effective in the presence of an ascorbic acid deficiency.[7] Szent-Györgi and his co-workers[8] demonstrated that the crude glucosides of citrin could control increased capillary fragility of guinea pigs. It is probable, although not proved, that the active part of the citrin is rutin. The relationship of ascorbic acid and rutin in the maintenance of capillary fragility is not known. Apparently only man, monkey and guinea pig, in marked contrast to dogs and mice, are dependent upon diet for ascorbic acid. On this basis it is probably desirable to repeat this work on guinea pigs, an animal whose dietary requirements for maintenance of capillary integrity are more nearly like that of man. However, there are many factors which influence survival following exposure to radiation, and capillary integrity may not be the critical one. Many animals die with only minor evidence of hemorrhage.[9,10] The control of the coagulation defect with protamine and toluidine blue in dogs by Allen[11] prevented hemorrhage to a great extent but the animals *died nevertheless.* The extensive use of blood and penicillin by one of us did not significantly influence the survival of goats exposed to the atomic bomb.[9] The present work conclusively demonstrates that rutin in the doses used was of no value in improving the survival of mice. The data presented suggest that rutin may have been harmful inasmuch as all rutin-treated groups died at a significantly faster rate.

Summary and conclusions. 1. Rutin not only was of no value in improving the survival of mice simultaneously exposed to a dose of ionizing radiation in the lethal range, but significantly increased the rate at which the mice died.

2. It is considered desirable to repeat this type of investigation on animals with ascorbic acid requirements similar to man.

The authors wish to acknowledge their appreciation for the rutin kindly supplied by J. F. Couch, Eastern Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, U. S. Department of Agriculture, Chestnut Hill Station, Philadelphia, Pa.

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16848 P

Increased Acetylcholine Sensitivity of Muscle by Acetone Magnification of the Rectus Test.

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It has been shown by Meng[1] that ketones and related substances sensitize the rectus muscle of the toad to acetylcholine (Ac). This sensitization is not correlated with inhibition of cholinesterase, and it is of the same degree whether the muscle is normal, eserinized or denervated. Hence the site of sensitization by these agents is said to be on the muscle. In connection with a study on the cholinesterase, we have found that acetone potentiates strikingly the action of Ac. The rectus muscle of the toad was recorded according to the standard technic.[2]

Fig. 1 compares the action of eserine and acetone on 2 recti of the same toad, showing the more striking potentiation by the latter.

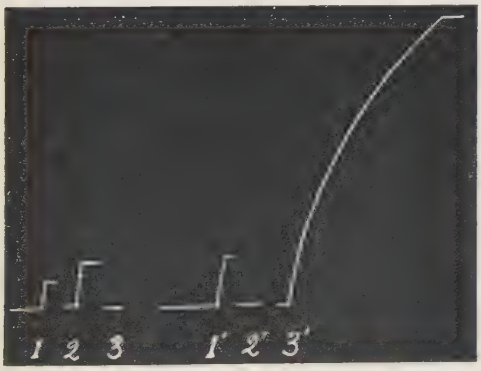

Fig. 1.

Comparing the sensitization of Ac by Es and acetone on the two recti of the same toad.

1. 1.38 γ Ac
2. 10 γ Es + 1.38 γ Ac } On right rectus
3. 10 γ Es

1′ 1.38 γ Ac
2′ 0.05 cc acetone } On left rectus
3′ 1.38 γ Ac + 0.05 cc acetone

Muscle bath = 8 cc. Each contraction recorded for 3 min.

Note that neither eserine nor acetone alone produced any contraction, while addition of Ac which by itself elicited only a weak response, caused a very marked contraction. The action of acetone could be repeated in succession if it was given each time. The concentration of acetone used had no influence on the cholinesterase as was shown by the fact that addition of known amount of Ac to the acetone-treated and control muscle mince showed comparable degree of hydrolysis.

Feng and Lee[3] have demonstrated that acetone can reproduce practically all the diverse actions of eserine on the skeletal N-M system. In the present experiment, we have shown the synergistic action of acetone and Ac, or probably the action of the former on the surface membrane favoring more ready penetration of the latter to the contractile elements of the muscle. Incidentally, we have made practical use of this striking response of Ac plus acetone in two ways: First, as an additional collateral test for qualitative identification of Ac. It is comparable to the leech test.[4] Secondly, as a quantitative assay for Ac in the range of 0.05 to 0.5 γ. Usually 0.05 to 0.5 γ Ac given to a bath of 8 cc will not touch the rectus muscle of the toad. If it is given with acetone, a significant contraction will be produced. This magnifying action of acetone makes it very useful for quantitative assay of Ac in the weak concentration defined. It is necessary that same amount of acetone must be added to the standard and unknown.

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16849

Influence of Detergents on Egg-White Inhibition of Hemagglutination by Formolized Swine Influenza Virus.*

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During the past year investigations have been made of the capacity of hen's egg-white (EW) to inhibit hemagglutination by purified swine influenza virus and derivatives of this virus obtained by heating or treatment with formaldehyde.[1,2] Many of the studies involved the use of purified, formolized swine influenza virus[3] which had been prepared for vaccine and stored for approximately 4 years at 4°C. The use of this preparation as a reagent for titrating inhibitor was encouraged since vaccine hemagglutination was found highly susceptible to EW inhibition,[1,2] and since the preparation could be regarded to have become stabilized during the prolonged storage.

Recently, certain irregularities in the results of these inhibition titrations have made it necessary to search for the factors concerned. This study has revealed a strong influence of soaps and synthetic ionic detergents on the inhibition reaction.

Materials and methods. The formolized virus (vaccine) employed in the present work, as well as the method of preparation of egg-white and the methods for estimation of hemagglutinative or inhibiting capacities, have been described in a previous report.[2] The details of significant deviations from the routine procedures will be included in the description of the experiments given here.

Experimental. The irregularities stimulating the present work may be illustrated by the results of a set of duplicate anti-vaccine inhibition titrations carried out on three successive days by the methods previously described.[2] Table I shows a titration, I*a*, characterized by excellent duplication, *steep* endpoint gradient, and *high* conventional (++) inhibition titer. Titration I*b* is characterized by excellent duplication, *shallow* endpoint gradient, and *low* conventional titer; however, inhibition, as shown by the occurrence of agglutination numbers smaller than the maximal number (++++), is evident in high EW dilutions. In contrast with these, titration I*c* shows irregularities which preclude any useful statement of titer. The analytically smooth character of titrations I*a* and I*b* suggested that the irregularities evident in titration I*c* were to be attributed to a factor other than the technic of the titrations.

During the period in which the initial investigations[1,2] were carried out, the titration tubes were cleaned in a solution of "green" soap (Medicinal Soft Soap, U.S.P.) and a commercial synthetic detergent (Orvus, Procter and Gamble). Later, hot chromic acid ("cleaning" solution) was substituted for the detergent mixture. Since the onset of the analytical irregularities was approximately coincident with this change in cleaning method, it appeared possible that extraordinary disagreement of duplicates might have resulted from the inadvertent use of tubes cleaned in the two different ways for the two members of a duplicate.

The experiment recorded in Table II was performed as a test. Titration II*a* was carried out in tubes cleaned with hot chromic acid, washed thoroughly in tap water followed by distilled water, and dried in the oven. Titration II*b* was carried out in tubes cleaned first with acid and dried, as above, and then cleaned again in green soap-Orvus mixture,

* This work was aided by a grant to Duke University from Lederle Laboratories, Inc., Pearl River, N. Y., and by the Dorothy Beard Research Fund.

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TABLE I.

Three EW Anti-Vaccine Inhibition Titrations,* in Duplicate, Illustrating the Analytical Irregularities Which Stimulated the Present Investigation.

Titration	Reciprocal of final EW dilution†						
	1,600	3,200	6,400	12,800	25,600	51,200	102,400
I*a*	0	0	0	+	++	+++	++++
(9/15/48)	0	0	0	+	++	+++	++++
I*b*	++±	++±	+++	+++	+++	+++±	+++±
(9/16/48	++±	++±	+++	+++	+++	+++±	++++
I*c*	+++	+++	+++±	+±	+++±	+++	+++±
(9/17/48)	++	+++±	+	+++	+++±	+++±	+++±

* Carried out at room temperature (28°C) with 4 HD (hemagglutinating doses) of vaccine. The EW dilutions and vaccine were incubated together for 40 minutes before RBC were added.

† Final EW dilution refers to the dilution of EW in the final reaction mixture including RBC.

TABLE II.

Influence of the Method of Cleaning Titration Tubes and of "Green" Soap on the EW Anti-Vaccine Inhibition Titration.

Titration	Cleaning method	Reciprocal of final EW dilution									
		200	400	800	1,600	3,200	6,400	12,800	25,600	51,200	†
II*a*	Acid	0	0	++	++±	++±	+++	+++	+++±	+++±	++++
		0	±	++	++±	++±	+++	+++	+++±	+++±	++++
II*b*	Detergent	0	±	+±	++	++±	++±	+++	++	+++±	++++
		0	±	++	±	++	++±	+±	+++±	+++±	++++
II*c**	Acid	0	0	0	0	0	0	0	+±	+++	++++
		0	0	0	0	0	0	0	+±	+++	++++

* Green soap was present in 1:80,000 (V/V) final dilution in all tubes.

† Indicates mixtures devoid of EW.

with thorough rinsing in tap and distilled water. Titration II*c* was carried out in acid-cleaned tubes in the presence of a constant, known amount of green soap; the soap was used in a final dilution, 1:80,000 (V/V), well below the hemolytic limit (see below), which was 1:8,000. Other experimental factors were held constant. A volume of 0.25 ml of buffered saline (II*a* and II*b*) or 0.25 ml of a 1:10,000 dilution of green soap in buffered saline (II*c*) was added first to the tubes, followed by 0.25 ml of the EW dilutions, which had been prepared in bulk in acid-cleaned tubes, and then by 0.5 ml vaccine dilution containing the customary 8 HD (hemagglutinating doses) per ml. These mixtures were incubated for 35 minutes at room temperature (25°C), and 1.0 ml of 2% chicken RBC was then added. After an additional hour at room temperature, the readings were made in the usual manner.[4]

Result II*a* (Table II) shows excellent duplication, shallow gradient, and low conventional titer. II*b*, while similar on the whole to II*a*, shows several poor duplications. II*c* shows excellent duplication, steep end-point gradient, and high conventional titer. From these results it is evident that the irregularities present in II*b* may be explained as deviations from the pattern of II*a* in the direction of II*c*, and, accordingly, it is reasonable to attribute the irregularity of II*b* to an irregular residuum of soap in the tubes used in this titration.

An explanation may now be given for results of the sort shown in Table I. It may be supposed that titration I*a* was carried out in tubes which had been cleaned with detergent and in which the residuum of detergent was

[4] Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., Beard, J. W., Feller, A. E., and Dingle, J. H., *J. Immunol.*, 1944, **48**, 129.

TABLE III.

Influence of "Green" Soap on the EW Anti-Vaccine Inhibition Titration; Dependence on Detergent Concentration.*

Reciprocal of final green soap dilution × 10⁻³	Reciprocal of final EW dilution									
	800	1,600	3,200	6,400	12,800	25,600	51,200	102,400	204,800	†
20	0	0	0	0	0	+	++±	+++±	++++	++++
40	0	0	0	0	0	+	+++	+++±	++++	++++
80	0	0	0	0	0	++	+++	+++±	++++	++++
160	0	0	0	0	0	++	+++	++++	++++	++++
320	0	0	0	0	±	++	+++	+++±	++++	++++
640	±	±	+±	+±	++	++±	+++	+++±	++++	++++
1,280	+	+±	++	++	++±	++±	+++±	++++	++++	++++
†	+	+±	++	++±	++±	+++	+++±	+++±	++++	++++

* For this experiment, EW dilutions and soap dilutions were mixed first, then 4 HD vaccine was added to each tube; after 30 minutes at room temperature (27°C), RBC were added. Mixtures devoid of EW or soap are indicated (†), representing an infinite dilution of the respective reagents.

great enough and uniform enough to cause considerable and uniform depression of the agglutination; that titration I*b* was carried out in tubes which had been cleaned with acid; and that titration I*c* was carried out in a mixture of the two kinds of tubes.

It was important to investigate next the dependence of the inhibition on the quantity of detergent present. Anti-vaccine inhibition titrations were carried out in acid-cleaned tubes in the presence of graded amounts of green soap or Duponol PC (du Pont), a synthetic detergent consisting chiefly of sodium dodecyl sulfate, with about 8-10% sodium sulfate and sodium chloride, a small amount of sodium tetradecyl sulfate, and perhaps traces of other alcohol sulfates.[5] Since the two detergents gave essentially the same results, only those obtained with green soap are presented (Table III). Reference to Table III shows that a significant effect was detectable with green soap in a final dilution of 1:640,000; with Duponol PC, a significant effect was observed with the highest dilution tested, 1:1,920,000. Furthermore, as the amount of detergent was increased, the effect on the inhibition increased rapidly at first and then more slowly until a concentration was reached beyond which the change was very gradual; at the same time, the titration gradient progressed from a shallow gradient at low detergent concentration to a steep gradient at high detergent concentration. Approximately the same plateau inhibition titer was attained with the two detergents; expressed as the reciprocal of the final EW dilution, this titer was approximately 40,000.

A calculation shows that the amount of detergent needed for a great effect on the inhibition is of the order of the amount which might reasonably remain in detergent-cleaned tubes after rinsing. Duponol PC, in a concentration of approximately 1 γ per ml of final reaction mixture, corresponding to a final dilution of 1:1,000,000, exerts a great, almost maximal, effect. Since the reaction mixture has a volume of 2 ml, the total quantity of detergent needed for this effect is 2 γ. The internal surface area of a titration tube of the sort used in the present experiments is approximately 30 cm^2. On the assumption that the detergent molecules have an average molecular weight of 300 and that each molecule can occupy 20 sq. Å of glass surface when close-packed with other molecules, the quantity of detergent needed to form one complete unimolecular layer on the glass may be calculated as $\frac{(30 \times 10^{16})\ (300 \times 10^{6})}{20\ (6 \times 10^{23})} = 7.5\ \gamma$. The amount of detergent, 2 γ, needed for a great detergent effect is thus approximately ¼ of the amount which will cover the internal surface of a titration tube with a single, close-packed unimolecular layer. A similar result may be calculated for green soap if reasonable assumptions are made about the composition

[5] Personal communication from Dr. J. H. Shipp of the Fine Chemicals Division, E. I. du Pont de Nemours and Company, Inc., Wilmington, Del.

of this complex material.[6] Since it is known[7] that in concentrated solution detergents exist as aggregates (micelles) in equilibrium with unassociated ions, it is possible that aggregates, as well as free ions, are adsorbed to the glass during treatment with concentrated detergent solution and that portions of these aggregates survive the after-rinsing. In this way an amount of detergent greater than the amount needed to form a unimolecular layer could be made available. Furthermore, the recorded effective concentrations of detergent are probably in excess of the actual concentrations, since an undetermined amount may be supposed to have adhered to the walls of pipettes and vessels other than the titration tubes during the preparation and distribution of the detergent dilutions.

It was of interest next to determine whether the detergent effect on EW anti-vaccine titrations could be demonstrated with detergents of different kinds. For this experiment, 0.5 ml vaccine (4 HD) was first placed in each tube, and then 0.25 ml of a dilution of detergent well beyond the hemolytic limit (see below) was added, followed after 15 minutes by 0.25 ml of two-fold EW dilutions. After a further period of 20 minutes at room temperature, 1.0 ml 2% RBC was added, and the agglutination readings were made as usual after one hour. The *hemolytic limit* of each detergent was determined by mixing 1.0 ml 2% RBC with 1.0 ml of each of several two-fold dilutions of detergent; the endpoint was taken as the highest dilution of detergent at which hemolysis could be detected visually after one hour at room temperature. The detergents not previously tested included the anionic detergents sodium oleate (Fisher, U.S.P.), Orvus (Procter and Gamble), Aerosol OT (American Cyanamid), and Sodium "Lorol" Sulfate PT† (du Pont), a purified, essentially salt-free detergent, consisting chiefly of sodium dodecyl sulfate; the cationic detergent Zephiran chloride (Winthrop-Stearns); and the non-ionic detergent Tween 80‡ (Atlas Powder). It was found that all of the ionic detergents exerted a great and strikingly similar effect on the EW anti-vaccine titrations; in other experiments, Zephiran chloride, which was noted to agglutinate RBC in high dilution of the detergent, gave irregular results which have not yet been further investigated. Tween 80, the only non-ionic detergent tested thus far, gave, in contrast with the other materials, a slight depression of inhibition, which has been confirmed but not further studied.

Discussion. A review of the data of Tables II and III shows that, in the range of concentrations in which they modify the EW inhibition of vaccine hemagglutination, the detergents, typified by "green" soap, do not inhibit hemagglutination by vaccine in the absence of EW; nor do they, in these concentrations, agglutinate RBC or cause hemolysis. On the other hand, EW, in the absence of detergent, gives evidence of *bona fide* inhibitor activity in high dilution, although the conventional (++) endpoint is reached in relatively low dilution. Indeed, examination of the results of many titrations indicates that, in general, EW and detergent together do not cause inhibition in EW dilutions appreciably beyond that dilution at which EW can be effective alone. Accordingly, we may provisionally interpret the detergent effect, which may be described as a sharpening of the inhibition endpoint, as an effect on the interaction of EW inhibitor and vaccine.

Summary. Investigation of the origin of irregular titration results has led to the observation that soaps and synthetic ionic detergents exert a great effect on the character of inhibition titrations involving purified, formolized swine influenza virus (vaccine) and the egg-white (EW) inhibitor of vaccine hemagglutination. The amount of detergent needed for such an effect is of the order of the amount which is sufficient to cover the internal glass surface of a titration tube with

6 Remington's Practice of Pharmacy, ninth ed., by Cook, E. F., and Martin, E. W., The Mack Publishing Company, Easton, Pa., 1948.

7 Putnam, F. W., *Advances in Protein Chem.*, 1948, **4**, 79.

† Obtained through the courtesy of Dr. J. H. Shipp of the E. I. du Pont de Nemours and Co., Wilmington, Del.

‡ Kindly furnished by Dr. Hilda Pope of the Duke University School of Medicine.

a single, close-packed unimolecular layer. While the detergent effect shows a dependence on detergent concentration, there exists a broad range of detergent concentrations over which the effect on the inhibition is essentially constant. For reasons indicated, the effect of detergent has been provisionally interpreted as an effect on the interaction of EW inhibitor and vaccine.

The mechanism of the detergent effect is being investigated and will be the subject of a subsequent report.

16850 P

Assay for Antistiffness Activity with Guinea Pigs Depleted on Solid Rations.

WALDEMAR DASLER* AND CLIFFORD D. BAUER. (Introduced by Richard G. Roberts.)

From Nutrition Research Laboratories, Chicago.

Oleson *et al.*[1] have described a solid basal ration with which they depleted guinea pigs for the purpose of conducting assays for antistiffness activity. Their results, however, seem to indicate shortcomings in the assay method. Thus, 13% of their negative controls showed marked improvement and an additional 17% showed slight improvement in stiffness. Furthermore, their homologous series of ergostanyl esters showed wide, inexplicable differences in activity.

Petering *et al.*[2] have recently described an apparently satisfactory assay method in which they used a solid, semipurified diet and with which they claim to have confirmed the antistiffness activity of ergostanyl acetate.

We have confirmed the production of wrist stiffness in guinea pigs with solid rations. Both the Lederle basal ration described by Oleson *et al.*[1] and the Rockland stock diet for guinea pigs† were successfully used to produce wrist stiffness. On the basis of our results, however, it is questionable whether the condition produced in guinea pigs on these rations is identical to the Wulzen stiffness syndrome which develops in animals fed the skim milk diets.[3–5] In our hands, in fact, these commercial, pelleted rations proved unsatisfactory for the assay of anti-stiffness activity.

That the condition which develops in guinea pigs on the Rockland and Lederle rations is not identical to that which has been produced by the Oregon group on skim milk diets is indicated by the fact that in our animals the easily hydrolyzable phosphorus of the liver did not decrease as drastically as in the experiments reported by van Wagtendonk[5] and van Wagtendonk and Wulzen.[6] In fact, we found no correlation between wrist stiffness and the easily hydrolyzable phosphorus of the liver in animals fed these rations.

We have nevertheless attempted to determine the antistiffness activity of a number of materials in assays involving several hundred animals fed these diets. Among the substances assayed were the previously reported active compounds listed in Table I.

Animals having a 3+ stiffness[1] were used for the therapeutic assays which, except for the diet, were conducted by the method of van Wagtendonk and Wulzen.[4,6] Supplements were dissolved in cottonseed oil and adminis-

* Present address: Department of Biochemistry, Chicago Medical School, Chicago, Ill.

1 Oleson, J. J., Van Donk, E. E., Bernstein, S., Dorfman, L., and SubbaRow, Y., *J. Biol. Chem.*, 1947, **171**, 1.

2 Petering, H. G., Stubberfield, L., and Delor, R. A., *Arch. Biochem.*, 1948, **18**, 487.

† Arcady Farms Milling Company, Chicago, Ill.

3 Wulzen, R., and Bahrs, A. M., *Am. J. Physiol.*, 1941, **133**, P500.

4 van Wagtendonk, W. J., and Wulzen, R., *Arch. Biochem.*, 1943, **1**, 373.

5 van Wagtendonk, W. J., *J. Biol. Chem.*, 1944, **155**, 337.

6 van Wagtendonk, W. J., and Wulzen, R., *J. Biol. Chem.*, 1946, **164**, 597.

TABLE I.
Therapeutic Tests—Lederle Basal Ration.

Supplement	Dose 5x/week μg	No. of animals	Stiffness after 7 weeks: Improved	Slight or questionable improvement	No change	Worse
Ergostanyl Acetate*	5	19	1	2	2	15
	50	8	3	2	1	2
Ergostanyl Acetate†	5	18	2	1	2	13
	50	5	0	2	1	2
Natural anti-stiffness Factor‡	50	9	4	1	1	3
None	—	9	1	1	2	5
"	—	8	3	2	1	2

* Prepared by Dr. O. N. Breivik, Standard Brands, Incorporated.
† Supplied by Lederle Laboratories.
‡ Supplied by Dr. W. J. van Wagtendonk.

TABLE II.
Ergostanyl Acetate*—8-Week Prophylactic Test—Rockland Diet.

Dose 7x/week, μg	No. of animals	Increase in stiffness: Marked	Slight	None
5000	10	4	1	5
500	10	6	3	1
5	6	3	2	1
0	15	6	2	7
0	15	10	3	2

* Supplied by Standard Brands, Incorporated.

tered orally. Because the results after 5 days were inconclusive, supplementation was continued. The assays were finally terminated after 7 weeks. The results are given in Table I. In this table weekly fluctuations in wrist stiffness are disregarded; only the over-all changes in stiffness which are manifest at the end of the 7-week period are indicated.

Prophylactic tests also were carried out with ergostanyl acetate on guinea pigs showing no stiffness and weighing 250-300 g at the start of the experiment. Our supply of the natural antistiffness factor was insufficient to permit its inclusion in this series. The results are shown in Table II. Some animals at all levels of ergostanyl acetate administration progressed through the whole range of wrist stiffness and, at the close of the eight-week experiment, had perfectly rigid wrists under the conditions of the test (1+ stiffness).

Summary and conclusions. Wrist stiffness was produced in guinea pigs by feeding two different commercial, pelleted rations. Both in therapeutic and in prophylactic tests, however, the untreated controls fared as well as the animals which received reputedly active supplements in amounts which have previously been shown to be more than adequate to give positive responses.[1,6] We interpret these results to indicate that (1) the mere production of wrist stiffness in guinea pigs does not necessarily yield animals satisfactory for assay purposes, that (2) the commercial, pelleted diets used by us and by Oleson *et al.*[1] are unsuitable as basal rations for the routine assay of the Wulzen antistiffness factor, and that (3) the activities of the steroids tested by Oleson *et al.* should be redetermined with animals depleted on more satisfactory basal rations.

16851

Inhibitory Action of Extracts of Mammalian Skin on Pigment Formation.

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Formation of melanin *in vitro* through oxidation of tyrosine by tyrosinase or by autoxidation of l-dihydroxyphenylalanine (dopa) is inhibited by skin extracts of rabbits[1,2] and of guinea pigs.[3,4] Rothman, *et al.*[5] have observed the inhibitory effect with extracts of isolated human epidermis. These authors advanced the theory that since they were able to counteract the inhibition with iodoacetamide, the inhibitory effect was due to the presence of sulfhydryl compounds in the epidermis. They suggested that pigmentogenic stimuli, such as ultraviolet rays, X rays, heat radiation and inflammatory processes caused pigmentation by oxidizing or destroying these inhibitory sulfhydryl compounds, thus enabling the enzyme to act on the pigment precursor.

The present work was carried out to investigate the nature and possible role of this inhibitory factor. The inhibitory action of aqueous extracts of human epidermis and of skin homogenates from rabbits was studied *in vitro*. In another series, rabbits were subjected to pigmentogenic stimuli and observations were made of the changes *in vivo*.

Methods. Aqueous extracts of human epidermis were prepared according to Rothman *et al.*[5] For preparation of extracts from rabbit skin, the hair on the back of the animals was clipped with scissors and on the bare skin two adjacent areas of equal size were mapped out with ink. One of the areas was covered with cloth, while the other was irradiated with an ultraviolet General Electric, RS, 275 W lamp. The distance varied from 6 to 12 inches and the exposure time from 5 to 15 minutes. Immediately after the irradiation the animal was killed. Skin samples were taken from the irradiated and non-irradiated areas. The subcutaneous fat tissue was removed and skin samples weighing from 0.3 to 0.5 g were frozen in dry ice and thawed rapidly several times in order to break up the cells. The pieces were minced and homogenized by means of glass homogenizers[6] with 2 ml distilled water in an ice bath, diluted with distilled water to a final concentration of 1 ml water for each 50 mg of tissue, placed in the ice box for 24 hours and centrifuged several times in the cold.

The inhibitory power of aqueous extracts of human epidermis and of the supernatant fluid of rabbit skin homogenates was determined by adding these extracts to buffered dopa solutions and by measuring the inhibition of melanin formation colorimetrically, as described elsewhere.[7] Sulfhydryl determinations were made with the ferricyanide method of Anson[8] in ultracentrifuged extracts and supernatant fluids of homogenates.

Results. In agreement with Rothman *et al.*[5] it was found that aqueous extracts of human epidermis inhibited the oxidation of tyrosine by tyrosinase and the autoxidation of dopa *in vitro*. As an index of the potency of the extracts the degree of inhibition of the autoxidation of dopa by 0.1 ml extract was chosen arbitrarily and the inhibition was ex-

* American Cancer Society Fellow, 1948-49.

1 Pugh, E. M., *Bioch. J.*, 1933, **27**, 475.

2 Danneel, R., and Schaumann, K., *Biol. Zbl.*, 1938, **58**, 242.

3 Schaaf, F., *Arch. f. Dermatol. u. Syph.*, 1938, **176**, 646.

4 Ginsburg, B., *Genetics*, 1944, **29**, 176.

5 Rothman, S., Krysa, H. F., and Smiljanic, A. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 208.

6 Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.

7 Flesch, P., PROC. SOC. EXP. BIOL. AND MED., in press.

8 Anson, M. L., *J. Gen. Physiol.*, 1941, **24**, 399.

pressed in percentages according to the formula:

$$\% \text{ Inhibition} = 100 \times \frac{(1\text{-colorimeter reading of tube containing extract})}{\text{colorimeter reading of control tube}}$$

Epidermis samples retained their inhibitory power for a considerable period of time when kept in the ice box before extraction. When the aqueous extracts were stored in the ice box, there was only slight diminution in the inhibitory effect. A fresh extract which caused 71% inhibition, showed at the end of 10 days 70%, and after 3 weeks 65% inhibition. Heating for 10 minutes in boiling water bath reduced the inhibitory effect by 10 to 40%. The inhibitory substance was completely dialyzed against distilled water through collodion membranes.

A few experiments were carried out with light brown extracts of 3 samples of Negro epidermis. No difference could be found between the extracts of epidermis from colored and white persons.

The inhibitory action of the extracts on dopa oxidation could be counteracted with p-chloromercuribenzoic acid, a specific sulfhydryl inhibitor. Cupric ion, a catalyst of sulfhydryl oxidation[9] and of the autoxidation of dopa,[10] also interfered with the inhibitory action of epidermal extracts. (Fig. 1).

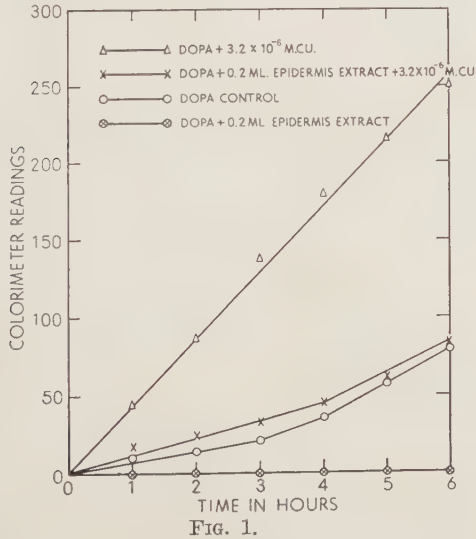

FIG. 1.

Inhibitory effect of human epidermal extract on autoxidation of dopa counteracted by cupric ions.

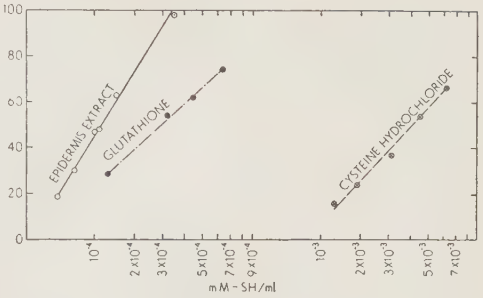

FIG. 2.

Relative inhibitory effect of cysteine, glutathione and extracts of human epidermis on autoxidation of dopa.

Estimation of the sulfhydryl content of the extracts gave values ranging from 4×10^{-5} mM/ml to 4×10^{-6} mM/ml. In most cases a correlation could be found between the sulfhydryl content of the extracts and their inhibitory power. When the -SH content was plotted on a logarithmic scale against the per cent inhibition, a straight line relationship was obtained. Such correlation was also found when glutathione (1.3×10^{-5} to 1.3×10^{-4} M final concentration) or cysteine hydrochloride (2.5×10^{-4} to 1.3×10^{-3} M final concentration) was added to dopa solutions. (Fig. 2). Calculated on the basis of the -SH content and in reference to 50% inhibition, the inhibitory effect of glutathione was about 13 times that of cysteine and the epidermis extracts were about 2.5 times more potent than glutathione.

When skin samples from human autopsy material were irradiated with ultraviolet light, no constant or reproducible differences could be observed between the -SH content of extracts of irradiated and non-irradiated epidermis. In order to eliminate the errors inherent in the preparation of human epidermis extracts (heating at 50°C for varying lengths of time, varying sizes of epidermal strips), living animals were irradiated and changes in the -SH content of the skin were studied in homogenates.

Colored rabbits are suitable for studies of pigmentation, because their hair is always

[9] Bernheim, F., and Bernheim, M. L. C., *Cold Spring Harbor Symp. Quant. Biol.*, 1939, **7**, 174.

[10] Flesch, P., *J. Invest. Dermatol.*, 1948, **11**, 157.

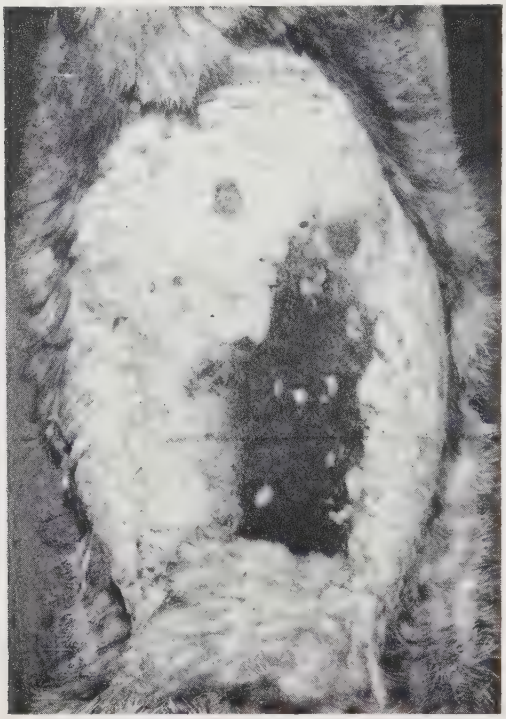

FIG. 3.
Pigmentation in grey rabbit after 12 days of rubbing daily for 1 minute with lanolin.

light colored near the skin. When the hair of colored rabbits is clipped and the bare skin subjected to irritation which leads to inflammation (heat, rubbing, ultraviolet light, etc.), the treated areas turn deeply pigmented after 1 to 2½ weeks and the regrowing hair becomes darker than it was before on the same area and than it is on the surrounding skin. The irritated areas are also characterized by exceedingly rapid regrowth of thick fur.[11-13] Clipping of the hair without irritating treatment leads primarily to an island-like regrowth of hair in pigmented areas[14] whereas hair growth in unpigmented spots is retarded.

In this laboratory one group of animals was rubbed daily for one minute with vaseline or lanolin over a clipped area on the back; the result in a grey rabbit after 12 days' rubbing is shown in Fig. 3. Another group of animals was irradiated daily for 10 minutes at a distance of 15 inches with the G.E. ultraviolet lamp. Pigment formation after ultraviolet irradiation was slightly more delayed than after rubbing. In experiments in which the -SH content of skin was determined, the rabbits were sacrificed immediately after a single irradiation was administered. In other animals one such treatment was found to cause pigmentation after 2 weeks latency period.

In 8 colored rabbits which were treated with ultraviolet light, the water extractable -SH compounds were markedly lowered in the aqueous extracts of homogenates obtained from irradiated skin samples as compared with similar extracts from non-irradiated skin. The average decrease was 53%. The results are summarized in Table I. Each result is the mean of three determinations, carried out in duplicate.

When the inhibitory power of these extracts was tested on the autoxidation of dopa, in 6 experiments the extracts obtained from irradiated skin showed 10 to 73% less inhibition than extracts from non-irradiated skin samples of the same animal. The average inhibition with extracts from irradiated skin was 29% and with extracts from non-irradiated skin 48%. (Fig. 4.)

Similar experiments were carried out on albino rabbits. In 6 experiments the amount of water-extractable -SH compounds did not show any significant change after irradiation. In 2 animals there was a decrease of 62 and 24% respectively.

On the basis of their -SH content, the extracts obtained from rabbit skin had about the same inhibitory power as aqueous extracts of human epidermis. No direct correlation could be found between the inhibitory power of the extracts and their -SH content.

Discussion. There are few data in the literature on the effect of ultraviolet light on sulfhydryl compounds of the skin. Keeser[15] found a marked reduction (50%) in the "reduced glutathione" content of rabbit skin after

[11] Lutz, W., *Arch. f. Dermatol. u. Syph.*, 1917, **124**, 233.

[12] Linser, K., *Klin. Wchschr.*, 1926, **5**, 1490.

[13] Linser, K., and Kähler, H., *Klin. Wchschr.*, 1928, **7**, 116.

[14] Königstein, H., *Arch. f. Dermatol. u. Syph.*, 1923, **143**, 314.

[15] Keeser, E., *Arch. f. exp. Path. u. Pharm.*, 1932, **166**, 624.

TABLE I.
Effect of Ultraviolet Light on the –SH Content of Pigmented Rabbit Skin.

Rabbit No.	Hair Color	Exposure time and distance		–SH expressed in 10^{-5} mM in		% decrease
		min.	in.	Non-irradiated skin extracts	Irradiated skin extracts	
1	Grey	10	6	10 ± 0.3*	1.7 ± 0.3	83
2	,,	15	8	15 ± 0.4	7.9 ± 0.1	47
3	Red-brown	15	12	39 ± 2.4	30.0 ± 1.3	23
4	,,	7	12	19 ± 1.2	12.0 ± 0.7	37
5	,,	15	12	18 ± 0.8	8.4 ± 2.1	53
6	Black	15	12	24 ± 2.3	4.6 ± 1.1	81
7	,,	15	12	16 ± 1.4	4.8 ± 0.9	70
8	,,	15	12	24 ± 1.8	9.2 ± 2.3	62

* Standard deviation of the mean.

TABLE II.
Effect of Ultraviolet Light on the -SH Content of Albino Rabbit Skin.

Rabbit No.	Hair color	Exposure time		–SH expressed in 10^{-5} mM in	
		min.	in.	Non-irradiated skin extracts	Irradiated skin extracts
1	Albino	15	12	11 ± 0.9	12 ± 2.3
2	,,	,,	,,	18 ± 1.2	21 ± 3.2
3	,,	,,	,,	17 ± 0.8	18 ± 2.8
4	,,	,,	,,	28 ± 1.6	30 ± 4.0
5*	,,	,,	,,	41 ± 2.1	43 ± 2.6
6	,,	,,	,,	26 ± 1.8	24 ± 3.1
7	,,	,,	,,	22 ± 1.4	8.5 ± 1.3
8	,,	,,	,,	17 ± 0.6	13 ± 1.1

* Skin homogenate was extracted for 48 hours.

ultraviolet irradiation, while others[16] noted a slight decrease (3%) up to one hour after irradiation. The color of the rabbits used in all these experiments has not been mentioned.

The finding that ultraviolet light decreases the amount of water-extractable -SH compounds in pigmented skin, supports the assumption that pigmentation after irritative stimuli is at least partly due to a decrease in the -SH content of the melanoblast. The observation that a decrease in -SH content does not occur after ultraviolet irradiation of albino skin, can be explained by assuming that most of the radiation passes through the albino epidermis, as it is the case in the mouse,[17] while in the pigmented skin the melanin in the basal layer prevents the radiation from reaching the corium. Absorption of ultraviolet light is therefore much greater in pigmented than in non-pigmented epidermis which is the site of the -SH compounds and of the inhibitory action.[4,18–21]

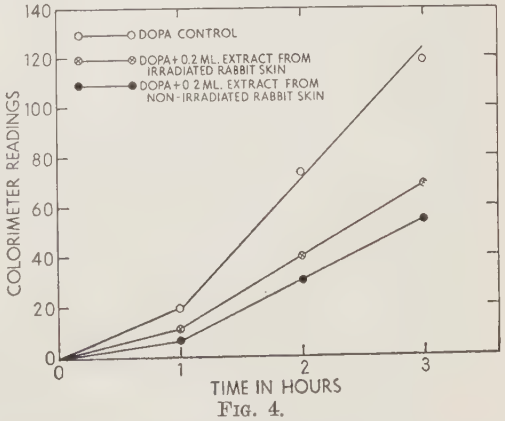

FIG. 4.

Inhibition of autoxidation of dopa with extracts of irradiated and non-irradiated rabbit skin.

16 Matusis, I. I., and Grechanovskii, V. P., *Bull. biol. med. exp. U.R.S.S.*, 1937, **3**, 489.

17 Kirby-Smith, J. S., Blum, H. F., and Grady, H. G., *J. Nat. Cancer Inst.*, 1942, **2**, 403.

18 Percival, G. H., and Stewart, C. P., *Brit. J. Dermatol. Syph.*, 1930, **42**, 215.

19 Giroud, A., and Bulliard, H., *La kératinisation de l'épiderme et des phanéres.* G. Doin, Paris, 1930.

20 Kaye, M., *Bioch. J.*, 1924, **18**, 1289.

21 Walker, E., *Bioch. J.*, 1925, **19**, 1085.

Summary. The inhibitory effect on melanin formation of aqueous extracts of isolated human epidermis and of homogenates of rabbit skin was found to be due to heat-stable, dialyzable, non-protein-like sulfhydryl compounds which were counteracted by cupric ions and p-chloromercuribenzoic acid. A direct relationship was found between the -SH concentration and the inhibitory power of extracts of human epidermis. Ultraviolet irradiation caused an immediate decrease in the amount of water-extractable -SH compounds of the skin of colored rabbits. No such decrease could be observed in albino animals. These findings support the previously advanced theory that pigment forming stimuli cause pigmentation by oxidizing or destroying the sulfhydryl compounds of the epidermis, whereupon the enzyme can freely act on the melanin precursor.

The author wishes to express his appreciation to Dr. Stephen Rothman for his valuable advice and interest in this work.

16852

Method for Determination of Sucrose and Sorbose in Blood and Urine.

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In this note is described a modification of the method of Hubbard and Loomis[1] for the determination of inulin which has proved satisfactory for the determination of sucrose and sorbose.

The method deviates from that described by these authors in the following ways.

The hydrochloric acid reagent is made by adding 5 volumes of concentrated HCl (sp. gr. 1.19) to one volume of distilled water. The resorcinol reagent is the same.

Plasma or serum filtrates are usually made in 1:20 dilution by precipitation with $Ba(OH)_2$ and $ZnSO_4$ according to the method of Somogyi.[2] Urine dilutions should be 1:100 or more. The one ml sample should contain between 30 and 160 μg of sucrose or between 20 and 150 μg of sorbose.

The procedure of Hubbard and Loomis is followed except for the following details. The test tubes are calibrated at 7.0 ml. After heating the mixture for 45 minutes in a water bath maintained at 70°C, 95% alcohol is added to the 7.0 ml mark, the solutions are mixed and are read in the Coleman spectrophotometer at a wave length of 530 mμ. In the case of sorbose, the density diminishes on standing; hence readings are made exactly 20 minutes after the end of heating.

Over the concentration ranges specified, the density was proportional to concentration for both sucrose and sorbose, sorbose being approximately 8% more chromogenic. Instead of running a blank we prefer to calculate a blank value by extrapolating the density of standards to zero content. In our experience the calculated blank density was usually less than 0.010.

Summary. The method of Hubbard and Loomis for the determination of inulin has been modified and adapted for the determination of sucrose and sorbose.

[1] Hubbard, R. S., and Loomis, T. A., *J. Biol. Chem.*, 1942, **145**, 641.

[2] Somogyi, M., *J. Biol. Chem.*, 1945, **160**, 69.

16853

Modification of Colorimetric Method for Determination of Mannitol and Sorbitol in Plasma and Urine.

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In this note is presented a modification of the method of Corcoran and Page[1] for the determination of mannitol which, in our hands, has proven simpler and more accurate than the original procedure. The method is applicable for the determination of sorbitol.

Reagents. 1. Periodic acid reagent: potassium periodate 0.0075 M in 0.25 M sulfuric acid.

2. Stannous chloride reagent: 0.035 M stannous chloride, reagent grade, in 0.33 M HCl. This reagent retains its strength for 4 days if kept in the refrigerator. After this time it results in blanks of high and variable density.

3. Chromotropic acid reagent. 0.15 g of chromotropic acid, purified by the method of Boyd and Logan[2] are dissolved in 20 cc of approximately 9 M sulfuric acid in a 200 cc volumetric flask. The contents are then made up to volume with concentrated (18 M) sulfuric acid. The first 20 to 30 cc of the concentrated acid should be added slowly, and with the flask in a cold water bath, to prevent heating the reagent. The density of the final reagent is approximately 0.010 at 570 mμ. It remains stable for many weeks if kept in the dark in a refrigerator.

4. Sulfuric acid, approximately 10.5 M.

Procedure. Mannitol-containing solutions should be preserved by the addition of a small amount of benzoic acid and kept at room temperature. Preservation in a deep freezing unit at —20°C may affect the determination by production of more color in the chromotropic reaction than corresponds to the liberation of 2 moles of formaldehyde per mole of mannitol.

Plasma filtrates are prepared as described by Somogyi.[3] Urines are diluted with water. The 2.0 cc sample should contain 7.0 to 40.0 μg of mannitol or sorbitol. The reagent blank consists of 2.0 cc of distilled water and is carried through the same procedure as the mannitol-containing solutions.

The procedure is carried out as follows: 2.0 cc samples of unknown, standards and water are pipetted into test tubes of 25 cc capacity. To each tube is added 0.5 cc of the periodic acid reagent and the contents are immediately and thoroughly mixed with a stirring rod. The tubes are then allowed to stand for 8 minutes at room temperature. The subsequent steps in the procedure should be carried out without interruption or delay.

After 8 minutes, 0.5 cc of stannous chloride reagent is added to each tube, the reaction mixture again well mixed, and the tubes are placed in a cold water bath. To each 5.0 cc of chromotropic acid reagent are added and the contents are mixed. It is advisable to add the chromotropic acid reagent to the blank before adding it to the mannitol-containing solutions. The tubes, with stirring rods in place, are immersed in a boiling water bath for 30 minutes. After removal and cooling, 10.0 cc of 10.5 M sulfuric acid are added to each tube, the contents are thoroughly mixed and the stirring rods removed.

The solutions are read in a Coleman spectrophotometer against distilled water at 570 mμ. The color is very stable, no change in density occurring if the tubes are permitted to stand for several hours before reading.

Calculations. Glucose, under the conditions described, is oxidized to formaldehyde to a slight and constant extent. By com-

1 Corcoran, A. C., and Page, I. H., *J. Biol. Chem.*, 1947, **170**, 165.

2 Boyd, M. J., and Logan, M. A., *J. Biol. Chem.*, 1942, **146**, 279.

3 Somogyi, M., *J. Biol. Chem.*, 1945, **160**, 69.

paring the densities of glucose-free mannitol standards with standards containing known amounts of glucose, the color production by glucose can be determined and correction for glucose content of the sample applied. In our experience the density per μg of glucose is 0.0024, as compared with 0.0142 per μg of mannitol. This value for glucose remains remarkably constant with varying proportions of glucose in the sample.

The density per μg of mannitol varies within $\pm$ 2% in the range of 10 to 30 μg of mannitol. For greater accuracy or for quantities beyond these limits, a calibration curve should be prepared.

Calculation of the mannitol content of the sample is as follows:

$$\frac{D_O - D_B - (K_G \times \mu g \text{ glucose in sample})}{K_m} = \mu g \text{ of mannitol in sample.}$$

Where D_O = observed density
D_B = density of blank
K_G = density per μg of glucose
K_m = " " " " mannitol.

Summary. A simple and accurate procedure for the determination of mannitol and sorbitol based on the method of Corcoran and Page has been described.

16854

Hemagglutinins in the Serum of Mice of Low and High Mammary Tumor Strains.

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The purpose of this communication is to report striking differences in the distribution of antisheep agglutinins in the serum of mice belonging to different inbred strains.

Preparatory to studying the response of inbred mice to antigenic stimulation, it became necessary to investigate natural hemagglutinins for sheep red cells, one of the antigens used. Gorer[1] reported the presence of isoagglutinins in mice, but no reference was found in the literature to natural heteroagglutinins in mice, although many investigations dealt with such natural antibodies in other species.[2]

Material. Healthy stock animals of 4 strains were used: 100 C57 black, 73 C3H, 123 dba, and 30 Marsh-albino animals.* The sex distribution was as follows: C57 black, 59 males and 41 females; C3H, 23 males and 50 females; dba, 32 males and 91 females; Marsh-albino, 9 males and 21 females. The age of the animals ranged from 10 weeks to 20 months, with the majority from 3 to 8 months. There was no apparent relation between the agglutinating ability of the mouse serum, and the sex or age of the animals, as represented in our material.

Technic. The animals were decapitated by a rapid stroke of sharp scissors. By holding the severed parts over a funnel, the blood was caught in small test tubes. The tubes were kept slanting until the blood clotted, left in the icebox for 18 to 24 hours, and then the serum was separated from the clots. The serum was inactivated in the water bath for 30 minutes at 56°C. The titration of the antisheep agglutinins followed the method used by the senior author in previous work.[3]

1 Gorer, P. A., *Cancer Research*, 1947, **7**, 634.

2 (a) Kolle and Wassermann, *Handbuch der pathogenen Mikroorganismen*, 1929, **3**, 351, 784; (b) Shimidzu, T., *Tohoku J. Exper. Med.*, 1932, **18**, 526.

* The animals were either bred in this laboratory from parent stock received 3 years ago through the courtesy of Dr. A. Tannenbaum, Michael Reese Hospital, Chicago, or were received directly from Dr. Tannenbaum.

TABLE I.
Distribution of Sheep Agglutinins.

	Strain							
	C57 Black		C3H		dba		Marsh-albino	
Titer	No.	%	No.	%	No.	%	No.	%
0	2	2	49	67.1	55	44.7	11	36.7
1	2	2	12	16.5	23	18.7	5	16.7
2	10	10	4	5.5	23	18.7	7	23.3
4	9	9	6	8.2	9	7.4	3	10
8	19	19	2	2.7	6	4.8	3	10
	Total	42		100		94.3		96.7
16	13	13	0		2	1.7	1	3.3
32	17	17	0		4	3.2	0	
64	15	15	0		1	0.8	0	
128	5	5	0		0		0	
256	4	4	0		0		0	
512	2	2	0		0		0	
1024	2	2	0		0		0	
	Total	58				5.7		3.3
Total No. of animals	100		73		123		30	

Sheep red blood cells, at least 24 hours old and not older than one week, were washed 3 times by centrifuging. The cells were not used unless the last supernatant was colorless. From the packed sheep cells a 2% suspension in physiologic solution of sodium chloride was prepared.

The test was set up as follows: Two drops of physiologic saline were added to each tube except the first. Two drops of serum were added to the first and second tube. After mixing, 2 drops of the second tube were transferred to the third tube, and so on. This gave serum dilutions of 1:2, 1:4, etc., which were continued according to the height of titer expected. One drop of the 2% sheep cell suspension was added to each tube and the rack was shaken. The tubes were incubated at room temperature for 2 to 3 hours. The reading of the agglutination was done grossly, and checked microscopically. Heavy "buttons" breaking up in large fragments were read as 3 plus; "buttons" breaking up in smaller grossly visible fragments, 2 plus; fine clumps visible under low-power magnification, 1 plus. The titer was given as the reciprocal of the highest dilution in which agglutination was found.

Results. Table I summarizes the titers obtained in the 4 inbred strains. Sheep agglutinin titers in C57 blacks reached by far higher values than those observed in any of the other strains. 58% of the C57 blacks presented titers of 16 or more, while among the C3H none of the titers exceeded 8; less than 6% of the dba animals and about 3% of the Marsh-albino exceeded this limit. 1024 was the highest titer observed in the C57 blacks; the highest titers in the C3H, dba and the Marsh-albino strains were 8, 64, and 16, respectively.

On the other hand, failure to agglutinate sheep erythrocytes in undiluted serum was found in only 2% of the C57 blacks, whereas 67.1% of C3H mice, 44.7% of dba animals, and 36.7% of Marsh-albinos showed titers of 0.

In order to study some of the properties of the antisheep agglutinins, pools of serum were prepared by sacrificing 9 to 11 animals of the C57 black strain. Preservation of the serum at 3-8°C for one month showed only insignificant changes in the agglutinin titer; after two months, usually a drop of the titer by 2 or more tubes was observed.

[3] Davidsohn, I., *J.A.M.A.*, 1937, **108**, 289.

TABLE II.
Absorption Experiments.

	Titer		
		After absorption with	
	Unabsorbed serum	Guinea pig kidney	Beef cells
Pool I	32	20	20
" II	64	40	40
" III	64	40	40

Table II gives the results of absorption of the serum with suspensions of guinea-pig kidney and of beef erythrocytes. The preparation of these antigens followed the technic given by the senior author.[4] 0.1 cc of the serum was mixed with 0.5 cc of the antigen suspension, and the mixture was kept for 1 hour at room temperature, with repeated shaking of the mixture. Following centrifugation, the supernatant was set up simultaneously with the unabsorbed serum. For the calculation, the dilution of the serum during the absorption was taken into consideration. Neither guinea-pig kidney nor beef red cells proved capable of removing the agglutinins from the serums to an appreciable degree.

Reading of the agglutination after increasing time intervals showed in some instances slightly higher titers after 2 hours of incubation as compared with the one hour titers; after 2 hours, the titers did not rise any further during observation for 24 hours. No significant change in titers was noted when the same serums were incubated at room temperature or 37°C. Occasionally, prolonged ice box incubation increased the titer by 1 or 2 tubes.

Instead of physiologic saline solution, albumin solution (20% bovine albumin) or serums of mouse, rat and rabbit were used as diluents for the serum to be tested and for the sheep cells. In the case of the animal serums, previous tests had shown the absence of antisheep agglutinins, or the serums were absorbed with sheep cells prior to use. In none of these experiments, did albumin or serum diluents raise the titer found with saline diluent; quite frequently, the titers in the albumin or serum dilutions were 2 to 3 tubes lower than the saline titers.

Table III lists agglutination tests which were set up with human O Rh-positive blood cells. The serum of 57 C57 blacks, of 61 C3H mice, and of 13 dba animals was tested. In marked contrast to the results obtained with sheep agglutinins, human red blood cells were clumped by less than 25 per cent of mouse serums and only in low titers. No appreciable difference was found between serums derived from animals of different strains. Nor was there any relationship between the simultaneously determined titer of sheep agglutinins, and the presence or absence of agglutinins for human erythrocytes.

In a few instances, also the agglutination of human erythrocytes of groups A and B (both Rh-positive) was tested with the serum of C57 black and of C3H mice. The number of experiments is too small to permit any conclusion. A cells showed a tendency to be clumped at a higher titer than O cells, but also here the titers were frequently far below those found in the same serum for sheep agglutinins.

Animals with spontaneous, transplanted and induced tumors were studied for the presence of hemagglutinins. The occurrence of antisheep agglutinins was not essentially different in tumor-bearing animals as compared with tumor-free animals of the same strains. A sufficiently large material to warrant definite conclusions is under investigation and will form the subject of a separate report.

Discussion. In Table IV the distribution of antisheep agglutinins is compared with the

TABLE III.
Agglutination of Human O Rh-positive Blood Cells By Mouse Serums.

	Strain		
Titer	C57 Blacks	C3H	dba
0	44	47	10
1	7	10	3
2	2	3	0
4	1	1	0
8	2	0	0
16	1	0	0
Total No. of Animals	57	61	13

[4] Davidsohn, I., and Walker, P. H., *Am. J. Clin. Path.*, 1935, **5**, 455.

TABLE IV.
Antisheep Agglutinins and the Incidence of Spontaneous Mammary Cancer in Inbred Strains of Mice.*

Strain	Authors	Tumor incidence, %	Antisheep agglutinins	
			Absent %	16 and higher, %
C3H	Andervont ('41)	91.4	67.1	0
	Bittner ('43)	92.3		
dba	Korteweg ('36)	76.3	44.7	5.7
	Murray & Hoffman ('41)	64.5		
Marsh-albino	Murray & Hoffman ('41)	76.4	36.7	3.3
	Haagensen & Randall ('42)	76.4		
C57 Black	Little, Murray, & Cloudman ('39)	0.5	2.0	58.0
	Haagensen & Randall ('42)	1.1		

* First 3 columns adapted from Walter E. Heston: Genetics of Mammary Tumors in Mice, in "A Symposium on Mammary Tumors in Mice," AAAS, 1945; p. 61, table I.

incidence of spontaneous mammary carcinoma, as recorded in the literature for the mouse strains furnishing the material of this study. It is apparent that the incidence of spontaneous mammary carcinoma in females in these strains is roughly inversely proportional to the presence and titers of the antisheep agglutinins. For instance, mice of the C57 black strain with extremely low mammary cancer incidence showed a very low percentage of serums lacking in sheep agglutinins and a high percentage of serums with titers of 16 and more. On the other hand, more than two-thirds of the serums of C3H mice, which possess the highest rate of tumor incidence, were completely lacking in agglutinins, and none of the animals so far examined showed a titer higher than 8. Animals of the dba strain, which develop mammary cancer quite frequently but less frequently than the C3H mice, lack sheep agglutinins in almost one-half of the cases, and less than 6% of the animals had titers of more than 8. Marsh-albinos, of which only a rather small number was tested, occupy a similar position in regard to tumor incidence; no agglutinins were found in more than one-third of the cases, and only 3.3% had titers higher than 8.

While no definite conclusions can be drawn from these findings at present, it may be hypothetically assumed that they are not coincidental but that the presence and the titer of the hemagglutinins are in some way connected with the genetic and other factors characterizing these mouse strains. In view of the fact that the incidence of spontaneous mammary carcinoma in the strains under discussion is determined by the milk factor and by hormonal influences in addition to genetic factors, those factors will have to be investigated.

The experiments on absorption of the agglutinins with guinea pig kidney suspensions showed that the agglutinins are not of the Forssman type; they also differed from the agglutinins found in serum of patients with infectious mononucleosis, since these latter are absorbed by beef red cells.[5] In this connection it may be pointed out that the position of the mouse in the Forssman system is not fully clarified. While older investigations placed this species into the "guinea pig group"[6] other workers presented contradictory evidence,[7] and more recent work showed differences between the true Forssman antigen and the antigens present in mouse tissues.[8] It is likely that especially the older investigations were carried out with heterozygous animals. For this reason studies on this

[5] Davidsohn, I., *Am. J. Clin. Path.*, 1938, **8**, 56.

[6] Doerr and Pick, *Biochem. Z.*, 1913, **50**, 129 (quot. 2a).

[7] Davidsohn, I., *Arch. Path. and Lab. Med.*, 1927, **4**, 776.

[8] Brown, G. C., *J. Immunol.*, 1943, **46**, 325.

problem are being carried out in this laboratory using inbred strains.

Previous work on the carmine storage in mice of inbred strains[9] demonstrated an inferior reticulo-endothelial storing ability in animals of the C3H strain as compared with C57 blacks. Although the origin and the significance of natural antibodies, such as the antisheep agglutinins studied in the present report, is not yet understood, there is considerable evidence in support of the part played by the reticulo-endothelial system in their production. In addition to the previously reported depression of the storing capacity of reticulo-endothelial tissues, the present study suggests impairment of the reticulo-endothelial system in relation to the production of natural antibodies in some mouse strains with high spontaneous mammary cancer incidence as compared with the resistant C57 black strain.

Summary. Heteroagglutinins for sheep erythrocytes were studied in the serum of 4 inbred mouse strains. Antisheep agglutinins were present much more frequently and in significantly higher titers in C57 black than in C3H, dba, and Marsh-albino strains. Some properties of the sheep agglutinins were studied. No such differences were found in the distribution of heteroagglutinins for human red cells in the different strains. The possible significance of these findings was discussed.

9 Stern, K., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 315.

16855

Nature and Probable Origin of Conjugated Histamine Excreted After Ingestion of Histamine.

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Anrep and coworkers reported that a histamine derivative appeared in the urine of various animals and a human subject after oral administration of histamine. This histamine derivative was inactive when tested on the isolated guinea pig ileum, but after acid hydrolysis of the compound a typical histamine response could be elicited. Injection of histamine was not followed by increased excretion of the derivative. Liver or intestine were suggested as likely sites for the conjugation of the ingested histamine.[1] Rosenthal and Tabor recently made the important observation that the chemical behavior of the urinary histamine derivative resembled that of acetyl histamine [4(β-acetyl aminoethyl) imidazole].[2]

The purpose of the present investigation was to test whether or not the histamine derivative is actually identical with acetyl histamine and to discover the origin of the compound in the body. The experiments described below present evidence, obtained by paper chromatography, for the identity of the histamine derivative with acetyl histamine and show that this compound appears in the feces as well as in the urine after oral administration of histamine. It is further shown that histamine is rapidly acetylated when added to fresh but not when added to previously autoclaved feces. Lastly, some common fecal microorganisms are shown to produce traces of acetyl histamine from histamine. The evidence obtained suggests that ingested histamine is acetylated within the intestinal contents from which it is partially absorbed and excreted by the kidneys. The methods employed are modifications of those previously developed for the identification of histamine

1 Anrep, G. V., Ayadi, M. S., Barsoum, G. S., Smith, J. R., and Talaat, M. M., *J. Physiol.*, 1944, **103**, 155.

2 Rosenthal, S. M., and Tabor, H., *J. Pharm. and Exp. Therap.*, 1948, **92**, 425.

in blood.[3]

Separation of acetyl histamine from related substances. A mixture of 10 μg of histidine, 10 μg of histamine and 10 μg of acetyl histamine was applied to 2 paper strips (Strips 1 and 2, Fig. 1) and the individual substances to separate strips (Strips 3, 4 and 5, Fig. 1). The chromatograms were developed for 15 hours with butanol saturated with 10% ammonium hydroxide as the mobile phase and 10% ammonium hydroxide saturated with butanol as the phase at the bottom of the chamber. After brief drying, color was developed by drawing the strips through a solution prepared by mixing 10 ml of a solution of 0.125% p-bromoaniline in 0.1 N hydrochloric acid with 10 ml of 3.7% sodium nitrite solution and adding to this mixture 10 ml of a 20% sodium carbonate solution. Histidine, histamine and acetyl histamine appear as red bands which are well separated (Fig. 1). (A number of other histamine derivatives were chromatographed in a similar manner. All of those tested migrated much faster than acetyl histamine.)

Approximate Rf values (Fig. 1)
Distance compound moves along paper
Distance solvent moves

Histamine		Acetyl histamine	
Strip 1	.56	Strip 1	.71
" 2	.56	" 2	.71
" 5	.55	" 3	.71

The Rf values for histidine are difficult to estimate because of its slow migration rate with the solvent used.

Urinary excretion of the histamine derivative after feeding histamine to a dog. A normal urine sample was collected from a healthy male dog who was then given histamine diphosphate, equivalent to 200 mg of the free amine, dissolved in 250 ml of water by stomach tube. The dog was sedated with morphine and atropine prior to the administration of the histamine solution to avoid vomiting.[2] Freshly voided urine was collected thereafter at intervals up to 33 hours.

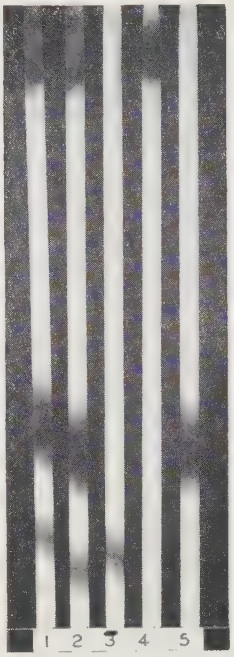

FIG. 1.

Separation of histidine, histamine, and acetyl histamine.

Strips 1 and 2: 10 μg histidine, histamine, and acetyl histamine applied as mixture.
Strip 3: 10 μg acetyl histamine.
Strip 4: 10 μg histidine.
Strip 5: 10 μg histamine.

From each sample of urine, 0.3 ml was diluted with water to 5 ml and 1.5 g of a salt mixture of 1 part $Na_3PO_4 \cdot H_2O$ and 6.25 parts anhydrous Na_2SO_4[4] was added. Two ml of butanol were added to the mixture and the tubes shaken for 20 minutes. After centrifugation, the butanol layer was applied to the paper strips by capillary pipettes.[5] Suitable controls were prepared by applying to strips extracts obtained from urine samples to which acetyl histamine and histamine had been added and by applying the pure substances to separate strips. The chromatograms were developed in the manner described in the foregoing experiment and uniformly showed bands in a position identical with that occupied by pure acetyl histamine on the control strips (Fig. 2). The chromatogram representing normal

3 Urbach, K. F., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 430.

4 McIntire, F. C., Roth, L. W., and Shaw, J. L., *J. Biol. Chem.*, 1947, **170**, 537.

5 Urbach, K. F., in press.

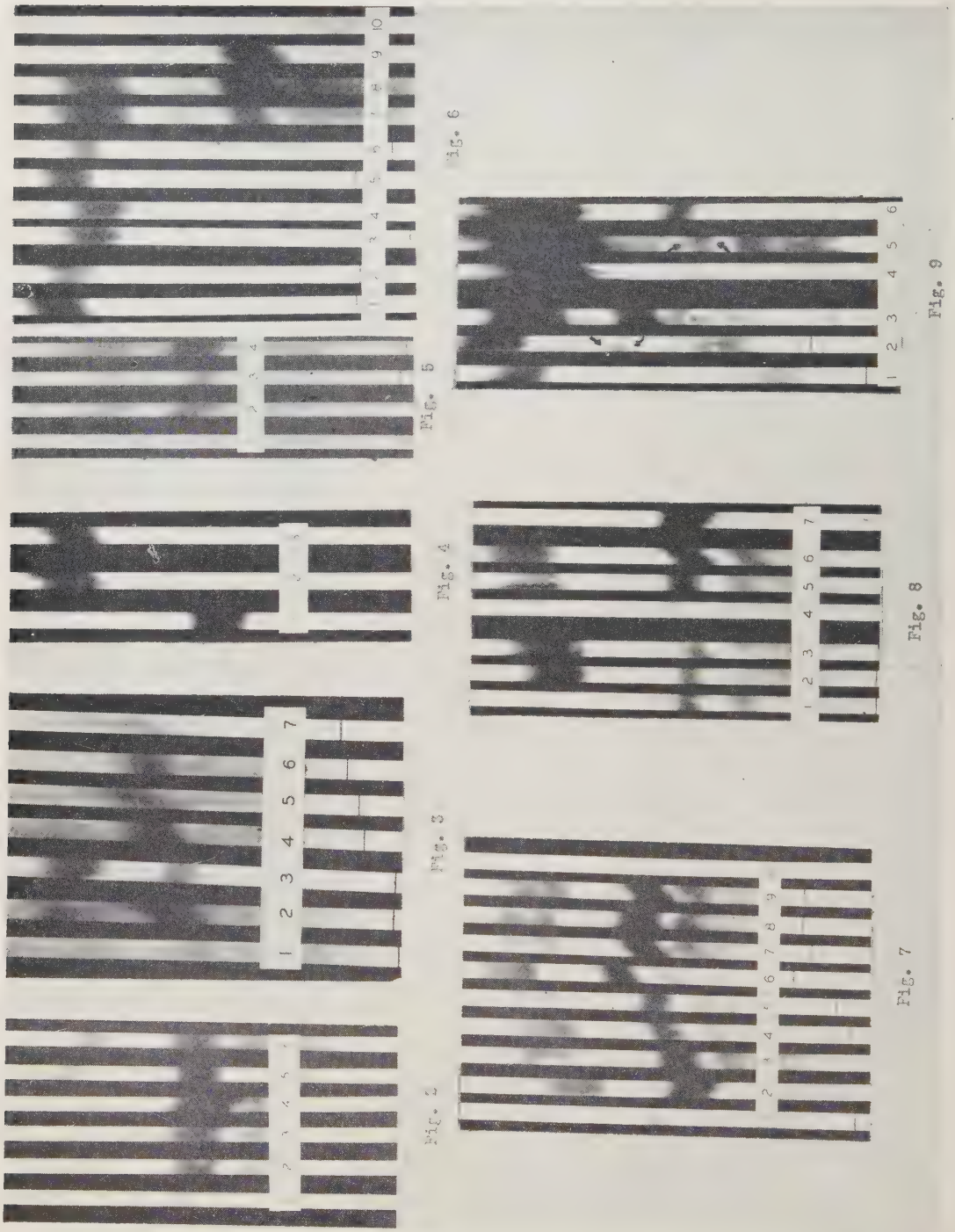

FIG. 2–9.

(All upper ends of the strips have been cut. The pencil lines on the lower ends of some of the strips indicate how far the solvent has advanced. On all chromatograms the upper bands are due to histamine and the lower due to acetyl histamine (histamine derivative) unless otherwise indicated).

FIG. 2—Chromatograms of dog urine after oral administration of histamine.

Strip 1—Normal urine.
" 2—Urine 22 hr after histamine administration.
" 3—Urine 33 hr after histamine administration.
" 4—Urine 33 hr after histamine administration, 20 μg acetyl histamine added.
" 5—Normal urine, 10 μg histamine and 10 μg acetyl histamine added.
" 6—Histamine and acetyl histamine (control).

FIG. 3—Chromatograms of human urine after oral administration of histamine.
Strip 1—Normal urine.
" 2—Urine 1½ hr after histamine administration.
" 3—Histamine and acetyl histamine (control).
" 4—Urine 3½ hr after histamine administration.
" 5—Urine 6 hr after histamine administration.
" 6—Urine 8 hr after histamine administration.
" 7—Urine 20 hr after histamine administration.

FIG. 4—Chromatograms of hydrolysis of acetyl histamine.
Strip 1—Acetyl histamine before hydrolysis.
" 2—Acetyl histamine after hydrolysis.
" 3—Histamine (control).

FIG. 5—Chromatograms of human stool after oral histamine administration.
Strip 1—Normal stool.
" 2—Stool 6 hr after histamine administration.
" 3—Stool 20 hr after histamine administration.
" 4—Normal stool plus acetyl histamine and histamine (control).

FIG. 6—Chromatograms of effect of histamine addition to bowel loops and bowel contents of a dog.
Strip 1—Washed ileum loop immediately after histamine addition.
" 2—Washed ileum loop incubated 4½ hr after histamine addition.
" 3—Ileum contents immediately after histamine addition.
" 4—Ileum contents incubated 4½ hr after histamine addition.
" 5—Washed colon immediately after histamine addition.
" 6—Washed colon incubated 4½ hr after histamine addition.
" 7—Colon contents immediately after histamine addition.
" 8—Colon contents incubated 4½ hr after histamine addition.
" 9—Acetyl histamine (control).
" 10—Colon contents before histamine addition.

FIG. 7—Chromatograms of effect of histamine addition to human stool.
Strip 1—Normal stool.
" 2—Histamine and acetyl histamine (control).
" 3-9—Stool immediately, 1 hr, 4 hr, 8 hr, 24 hr, 30 hr, and 55 hr after histamine addition and incubation.
" 10—Normal stool 24 hr after incubation.

FIG. 8—Chromatograms of effect of autoclaving human stool on acetyl histamine formation.
Strip 1—Autoclaved stool.
" 2—Autoclaved stool immediately after histamine addition.
" 3—Autoclaved stool incubated 4½ hr after histamine addition.
" 4—Nonautoclaved stool.
" 5—Nonautoclaved stool immediately after histamine addition.
" 6—Nonautoclaved stool incubated 4½ hr after histamine addition.
" 7—Acetyl histamine (control).

FIG. 9—Chromatograms of effect of addition of histamine to pure cultures of *Aerobacter aerogenes* and *E. coli*.
Strip 1—Aerogenes culture immediately after histamine addition.
" 2—Aerogenes culture incubated 40 hr after histamine addition.
" 3—Histamine and acetyl histamine (control).
" 4—*E. coli* culture immediately after histamine addition.
" 5—*E. coli* culture incubated 4½ hr after histamine addition.
" 6—Histamine and acetyl histamine (control). (Traces of acetyl histamine at arrows. On Strip 5 at least 2 unidentified bands appear below the acetyl histamine).

urine shows the presence of a small amount of the histamine derivative (Strip 1, Fig. 2).

Approximate Rf values (Fig. 2)

Histamine		Urinary histamine derivative		Control acetyl histamine	
Strip 5	.55	Strip 1	.73	Strip 5	.74
" 6	.57	" 2	.73	" 6	.73
		" 3	.73		

Urinary histamine derivative with acetyl histamine added	
Strip 4	.73

Urinary excretion of the histamine derivative after ingestion of histamine by a human subject. After collecting a sample of normal urine, a gelatin capsule containing 100 mg histamine diphosphate was given by mouth to a human subject (K.F.U.). Urine was collected at frequent intervals after the ingestion

of histamine. Since the amount of conjugate present was small, the size and purification of the samples had to be modified: to 20 ml of urine were added 6 g of the salt mixture described. The samples were extracted twice with 10 ml of butanol. The combined butanol extracts were aerated until free from ammonia and were then passed through cotton succinate.[4] After passage of the butanol extracts, the cotton succinate was washed with 2 ml of acetone which was followed by elution with 2 ml of 0.5 N H_2SO_4, followed by 2 ml H_2O. The combined H_2SO_4-H_2O eluate was neutralized with 10 per cent NaOH and 1.5 g of the salt mixture of Na_3PO_4 and Na_2SO_4 was added. The resulting solution was extracted with 2 ml of butanol, which was then applied to the paper strips as in the previous experiment. Control strips were prepared as in the foregoing experiment. The amounts of histamine derivative appearing on the chromatograms are much smaller than those observed in the previous experiment. This is partly explained by the smaller amount of histamine employed and partly by the failure of cotton succinate to absorb acetyl histamine quantitatively.[2] Moreover, Anrep has reported that man, after the oral administration of histamine, does not excrete as much of the histamine derivative in the urine as does the dog.[1] It is of interest to note that free histamine, as well as histamine derivative, appears in the human urine early after the histamine administration (Strip 2, Fig. 3).

Approximate Rf values (Fig. 3)

Histamine		Control acetyl histamine		Histamine derivative	
Strip 3 (control)	.53	Strip 3	.69	Strip 2	.69
Strip 2	.53			Strip 4	.68
				Strip 5	.68
				Strip 6	.70

Hydrolysis of acetyl histamine to histamine. The foregoing experiments provide evidence that the substance excreted in the urine of dog and man after histamine ingestion is acetyl histamine. In order to obtain evidence for the identity of the compound described by Anrep[1] with acetyl histamine, a sample of synthetic acetyl histamine was hydrolized under the conditions used by Anrep for the hydrolysis of the urinary compound to histamine. Six mg of acetyl histamine were dissolved in 5 ml of water and 1 ml concentrated hydrochloric acid. 0.01 ml (10 μg) of the solution was applied to paper strips both before and after boiling the solution for one and one-half hours. A control strip was prepared by applying 10 μg of histamine. Chromatograms were prepared as in the first experiment, and are shown in Fig. 4. It is evident that acetyl histamine is easily converted to histamine under the conditions employed. A trace of acetyl histamine was still present after the hydrolysis, possibly because of the relatively large amount of acetyl histamine employed. The approximate Rf values of pure histamine (Strip 3) is 0.55 and of histamine obtained from the hydrolysis of acetyl histamine (Strip 2) 0.55.

Excretion of the histamine derivative in human feces after oral administration of histamine. The previous experiments presented evidence for the identity of the urinary histamine derivative with acetyl histamine. Because of the possibility that the histamine derivative might also appear in the feces, as well as the urine after feeding histamine, 3 stool samples were collected before, 6 and 20 hours after the administration of the histamine to the human subject. To 1 g (wet weight) of each sample was added 10 ml water. A fourth sample was prepared by adding 20 μg of each histamine and acetyl histamine to 1 g of the stool sample collected before the histamine administration. All samples were shaken, filtered and 3 g of the previously mentioned salt mixture was added to the filtrates which then were twice extracted with 10 ml portions of ether. This was followed by extraction with 2 ml of butanol which were passed through cotton succinate. The elution of the cotton succinate and the preparation of the chromatogram shown in Fig. 5 were carried out as in the third experiment. Strip 1 (normal stool) shows no band. Strip 2, representing the stool sample obtained 6 hours after the ingestion of histamine, shows a pronounced acetyl histamine band below and a weak histamine band above. Strip 3, corresponding to the stool sample taken 20 hours after the ingestion of histamine, shows a weaker acetyl histamine band and only a very

faint trace of histamine. Strip 4 represents normal stool with histamine and acetyl histamine added.

Approximate Rf values (Fig. 5)

Histamine		Histamine derivative in stool		Control acetyl histamine	
Strip 2	.57	Strip 2	.71		
Strip 3	.57	" 3	.72		
Strip 4	.57			Strip 4	.70

Formation of the histamine derivative after addition of histamine to isolated intestinal loops and bowel contents of a dog. The appearance of the histamine derivative in feces, described in the previous experiment, could be due to two different mechanisms. Either the histamine derivative is formed inside the intestine or histamine is absorbed from the intestine, changed and re-excreted into the contents of the bowels. Since the histamine derivative had not been observed in the urine after the injection of histamine,[1] the first possibility was assumed to be more likely. Since it was, therefore, of interest to investigate whether the derivative is formed through interaction of histamine with the intestinal wall or with intestinal contents, the intestines were removed from a freshly killed dog and the contents were removed from segments of colon and ileum which were then washed clean and tied. The loops were filled with 10 ml of saline containing 1 mg of histamine per ml. To the intestinal contents 1 mg histamine per gram was added after removal of 1 g samples. All preparations were incubated at 37°C and 1 ml or 1 g samples were withdrawn after appropriate intervals. Each sample was diluted to 20 ml with water. The mixture was shaken and centrifuged and chromatograms were prepared from 1 ml of the supernatant by diluting to 5 ml, adding salt mixture, ether and butanol extraction and cotton succinate purification as described in the previous experiments. The chromatograms illustrated in Fig. 6 show that the histamine derivative was formed rapidly in the contents taken from the colon, but not in the washed loops in which histamine disappeared without formation of acetyl histamine. In the contents taken from the ileum traces of the histamine derivative were formed.

Approximate Rf values (Fig. 6)

Control acetyl histamine		Histamine derivative	
Strip 9	.74	Strip 7	.75
		" 8	.75

Formation of the histamine derivative after addition of histamine to human feces. The preceding experiment suggested intestinal contents as a site for the conversion of histamine. It was, therefore, of interest to investigate if human feces could convert histamine in a similar manner. Freshly passed human feces were divided into 2 samples. To one sample 1 mg/g histamine was added and 1 g portions were taken immediately from the control and histamine-containing feces. Both samples were then incubated and 1 g portions were withdrawn at convenient intervals. Chromatograms were prepared as described in the previous experiment and are shown in Fig. 7. Strip 1 (normal stool) shows a small amount of the histamine derivative present before histamine addition. Strip 2 represents 10 μg acetyl histamine and 10 μg histamine added to normal stool as a control and the following strips represent stool samples taken at various times after the histamine addition. It is of interest to note the appearance of an unidentified band (Rf 0.81) after 30 and 55 hours incubation. Strip 10 represents normal stool after 24 hours incubation.

Approximate Rf values (Fig. 7)

Control acetyl histamine		Histamine derivative	
Strip 2	.72	Strip 3	.71
		" 4	.70
		" 5	.70
		" 6	.70
		" 7	.71
		" 8	.72
		" 9	.72

The influence of autoclaving human feces on the formation of the histamine derivative. The previous experiment showed that histamine, when added to fresh human feces, is converted effectively to acetyl histamine. In order to test whether the reaction is due to a biological process or due to a chemical reaction not requiring living matter, a freshly passed human stool specimen was divided into 2 portions, one of which was autoclaved at 200 lb pressure for 20 minutes. After taking 1 g samples from the normal and autoclaved portions, 1 mg histamine per gram of stool was

added to each portion. One gram samples were withdrawn immediately after mixing the histamine into the stool and again after 4 hours incubation. The chromatograms shown in Fig. 8 were prepared as described above. The autoclaved sample, before the addition of histamine, shows a faint band, due to the histamine derivative which does not increase in intensity after incubation with histamine (Strips 1, 2, 3). The bands due to the histamine derivative increase markedly in color intensity on incubation of the non-autoclaved sample after addition of histamine (Strips 4, 5, 6). Pure acetyl histamine was applied to Strip 7 as a control.

Approximate Rf values (Fig. 8)

Control acetyl histamine		Histamine derivative	
Strip 7	.71	Strip 1	.73
		" 2	.73
		" 3	.73
		" 4	.72
		" 5	.71
		" 6	.71

Attempts to convert histamine to acetyl histamine with pure cultures of E. coli and Aerobacter aerogenes. The experiment showing the formation of the histamine derivative made it appear likely that conversion of the histamine was due to fecal organisms. Histamine, was, therefore, added to a pure culture of *E. coli* and of *Aerobacter aerogenes* incubated in a simple glucose-salt medium.[6] Samples of the cultures were withdrawn at intervals and chromatograms, shown in Fig. 9, were prepared in a manner similar to that described in the previous experiments. Both organisms appear to form acetyl histamine in small quantities from added histamine. In addition, the organisms apparently are able to produce other unidentified substances from histamine which give colored bands with the color reagent employed.

Approximate Rf values (Fig. 9)

Control acetyl histamine		Acetyl histamine formed in culture	
Strip 3	.70	Strip 2	.71
" 6	.71	" 4	.70

Discussion. The experiments presented provide evidence that the histamine derivative recoverable from urine and feces of dog and man is acetyl histamine.* This substance is probably identical with the histamine derivative described by Anrep.[1] The fact that acetyl histamine is formed from histamine in normal but not in autoclaved feces and the ability of common fecal organisms to form traces of acetyl histamine in pure culture suggests that the observed acetylation of histamine in feces may be due to bacteria. The fact that *E. Coli* and *Aerobacter aerogenes* in pure culture are not able to produce acetyl histamine as effectively in quantities comparable to those obtained from feces may be due to several obvious differences in the conditions obtaining in stool samples and in simple culture media. Other fecal organisms may be able to acetylate histamine much more effectively. The possibility that histamine may also be acetylated elsewhere in the body, *e.g.* the liver, has been suggested and is not excluded by the present experiments. It should be noted that on occasion small quantities of acetyl histamine and histamine were observed in normal stool and urine samples.

Conclusions. 1. The observation that a histamine derivative appears in the urine of dog and man after oral histamine administration is confirmed.

2. The histamine derivative can be effectively separated from histamine by paper chromatography.

3. The histamine derivative is identical with acetyl histamine as shown by paper chromatography.

4. Acetyl histamine is readily formed from histamine added to feces of dog and man.

5. It is suggested that histamine is acetylated in the intestinal contents to acetyl histamine which is partially absorbed from the intestinal tract and excreted in the urine as well as in the feces.

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16856

Chemistry of the Liver Cytoplasm of Normal, Fasted and Cirrhotic Mice.

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An impairment of the mechanism for the mobilization of lipide material from the liver has been reported to be present in both starvation[1] and cirrhosis.[2] MacLachlan[1] has observed that the liver of fasted albino male mice accumulates neutral fat in such amounts, that in the first 2 days, the quantity of total lipide increased 2 to 3 fold. As the starvation is continued, the amount of total lipide decreases even below that of the normal, and the phospholipides show a downward trend during this entire period of starvation. Wright[2] observed that the alcoholic cirrhotic liver is pale, enlarged, and most of the liver cells are distended with fat. This lipide may result from the inadequate diet observed in many cirrhotics. The liver cell has a cytoplasm which appears to be quite labile,[3] so that an investigation of the chemical changes occurring in this portion of the cell, during cirrhosis and fasting, might give some indication as to the integral processes involved.

Three experimental groups of animals were studied: 1. Normal, fully fed animals. 2. Animals which were fasted for 24 hours, and 3. Cirrhotic animals which were fasted for 24 hours.

Materials and methods. Male mice, 5 to 7 months of age, of the C_3H strain were utilized in this experiment. Hepatic cirrhosis was produced by feeding 0.1 ml of 40% carbon tetrachloride in olive oil to mice every 4 days for a period of 200 days.[4,5] Cirrhosis was confirmed by histologic examination.†

Livers, of mice anaesthetized with ether, were removed after clamping the portal vein and cutting the hepatic vein. The blood that was present in the liver was thus allowed to drain.

The cytoplasmic extract was prepared essentially according to the method of Claude.[6] Livers from 10 mice were pooled for each group, ground in a mortar, suspended in 4 volumes of alkaline saline (2 ml 0.1 N NaOH per liter of physiologic saline), then spun at 1400 × g in the Sorvall angle centrifuge. The decanted supernatant fluid was considered to contain a major aliquot of the total cytoplasm. Lipides that came to the surface in the various preparations were resuspended in the extract before the analyses were performed. It was assumed that all of the lipide that rose to the surface belonged to the cytoplasm, since Rosenfeld[3] observed that an increase of lipide during various pathologic conditions, *e.g.* phosphorus poisoning, was purely cytoplasmic in nature.

Separate aliquots were removed from the cytoplasmic extract for the determination of total nitrogen,[7] phosphorus,[8] "ribonucleic acid," and lipide. Total solids were obtained by heating an aliquot of the cytoplasmic extract in a tared weighing bottle over a boiling water bath until the fluid from the extract disappeared. The bottles were then placed in an oven at 105°C for one hour.

The "ribonucleic acid" was determined essentially by Brown's modification[9] of the

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TABLE I.
Chemistry of the Liver Cytoplasm of Normal, Fasted, and Fasted-Cirrhotic Mice.*

Experiment†	Mg per g wet weight liver					
	Total solids	N	P	"RNA"	N/P	N/"RNA"
Fully fed	206	22.2	2.54	11.2	8.75	1.98
	235	29.1	2.68	12.1	10.9	2.41
Fasted	294	24.1	2.47	11.3	9.78	2.13
	377	30.5	3.09	14.2	9.88	2.15
Fasted	213	23.4	2.28	7.26	10.2	3.22
Cirrhotic	241	27.0	2.46	8.18	10.9	3.31

* It was assumed that nearly all of the cells were ruptured[13] and the concentration of the chemical constituents, *e.g.*, N, P, "RNA", total lipide, and phospholipide in the cytoplasmic extract, was the same as their concentration in the original ground tissue. With this assumption it is possible to consider the chemistry on a wet weight basis and thus avoid the discrepancy that may arise in dry weight due to the large amounts of lipide material present.

† Each figure results from the analysis of one pool of 10 animals.

TABLE II.
Lipide Chemistry of the Liver Cytoplasm of Normal, Fasted, and Fasted-Cirrhotic Mice.

Experiment†	Mg per g wet weight liver			
	Total lipide	Phospholipide	N/P (atomic)	Iodine value
Fully fed	43.4	22.0	1.10	125
	46.5	22.2	1.24	129
Fasted	140	23.9	1.41	92.5
	177	25.2	1.48	95.5
Fasted	67.5	16.1	1.50	90.2
Cirrhotic	81.5	17.4	1.64	91.8

* Each figure results from the analysis of one pool of 10 animals.

orcinol-HCl method in which the pentose values are corrected for the presence of hexose. The "ribonucleic acid" was calculated by multiplying the total tetranucleotide ribose by the factor 2.14.

Lipide was extracted with Bloor's solution (alcohol:ether; 3:1). Bloor's solution was re-extracted with petroleum ether, care was taken to wash the lipide extract with water to remove any of the non-protein nitrogen contaminants.[10] A weight of lipide was obtained by evaporating the petroleum ether under reduced pressure from tared 10 ml volumetric flasks. The iodine value was determined by the method of Kretchmer *et al.*[11]

Experimental results. Table I shows the actual amount of nitrogen, phosphorus, and "ribonucleic acid" present in the liver cytoplasm obtained from one gram wet weight of liver. There are no differences between the various liver cytoplasms as concerns nitrogen, but the phosphorus appears to have decreased in the fasted-cirrhotic liver which is probably in part due to the decrease observed in the "ribonucleic acid" in these livers. This decrease in "ribonucleic acid" is obviously not due to the fast but to the cirrhosis since there is no decrease in "ribonucleic acid" in the fasted livers.

Total lipide (Table II) increases approximately 3 to 4 fold after a 24 hour fast, but this excess lipide seems to be entirely non-phospholipide in character since the phospholipide remains constant. The lipide phosphorus to nitrogen ratio indicates that the phospholipide present in the cytoplasm has been altered in that a lipide of a higher nitro-

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gen content has entered the lipide extract. Cirrhosis affects the lipide content so that it increases above normal but not as high as the normal fasted liver cytoplasm. The phospholipide in the cirrhotic liver decreases, but here also there seems to be a lipide present with a high nitrogen content.

The iodine value of both the normal fasted and the cirrhotic-fasted liver cytoplasms decreased below that of normal. These results are in accord with those of Winter[12] in that he found that after feeding carbon tetrachloride to rats the iodine value of the liver fatty acids decreased from 111 to 100.

Summary and discussion. Chemical changes in the cytoplasm of the mouse liver cell after a short fast and at cirrhosis are reported. These changes occur most dramatically in the lipide fraction during a 24 hour fast resulting in an increase of 3 to 4 fold above normal. This lipide is non-phospholipide in character. Fasted cirrhotic liver differs from the fasted normal liver cytoplasm in that the lipide does not increase so markedly and the phospholipide decreases to about 60% of the normal fasted value. The iodine values of the normal-fasted and the cirrhotic-fasted liver cytoplasm fatty acids decrease below that of the normal values.

In addition to a lipide increase in the fasted-cirrhotic liver cytoplasm there is also a decrease of "ribonucleic acid" and phospholipide in one gram wet weight of the ground fasted cirrhotic liver.

It is possible that there is an interchange of phospholipide during a fast as evidenced by the lipide nitrogen to phosphorus ratio. This interchange would consist of substituting normal phospholipide with a lipide or phospholipide which contains a great deal of nitrogen.

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16857 P

Electrical Alternation in Experimental Coronary Artery Occlusion.

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The purpose of this preliminary note is to report a high incidence of electrical alternation in experimental coronary artery occlusion.

Methods. In open-chested nembutalized dogs,[1] the left anterior descending coronary artery was occluded for periods of 30 seconds to 32 minutes. Electrocardiographic tracings were made with the exploring electrodes in the cavity of the left ventricle and on the epicardial surface or precordium. Thirty-three experiments were performed on 11 dogs.

Results. Electrical alternation occurred in 8 of 9 dogs (89%) which developed electrical signs of myocardial ischemia[2] following coronary artery occlusion. One dog died of ventricular fibrillation, and one dog failed to develop any electrical signs of ischemia following occlusion for 32 minutes. Electrical alternation occurred about 2 minutes after occlusion, was variable in duration (several seconds to 20 minutes), and disappeared either spontaneously or within 3 seconds to 5 minutes after release of the constriction. Alternation of the ST-T and QRS complexes, alone or together, was observed, the most common being of the ST segment (Fig. 1). There was alternation in the extent of ST elevation in the epicardial leads, and of ST segment elevation or depres-

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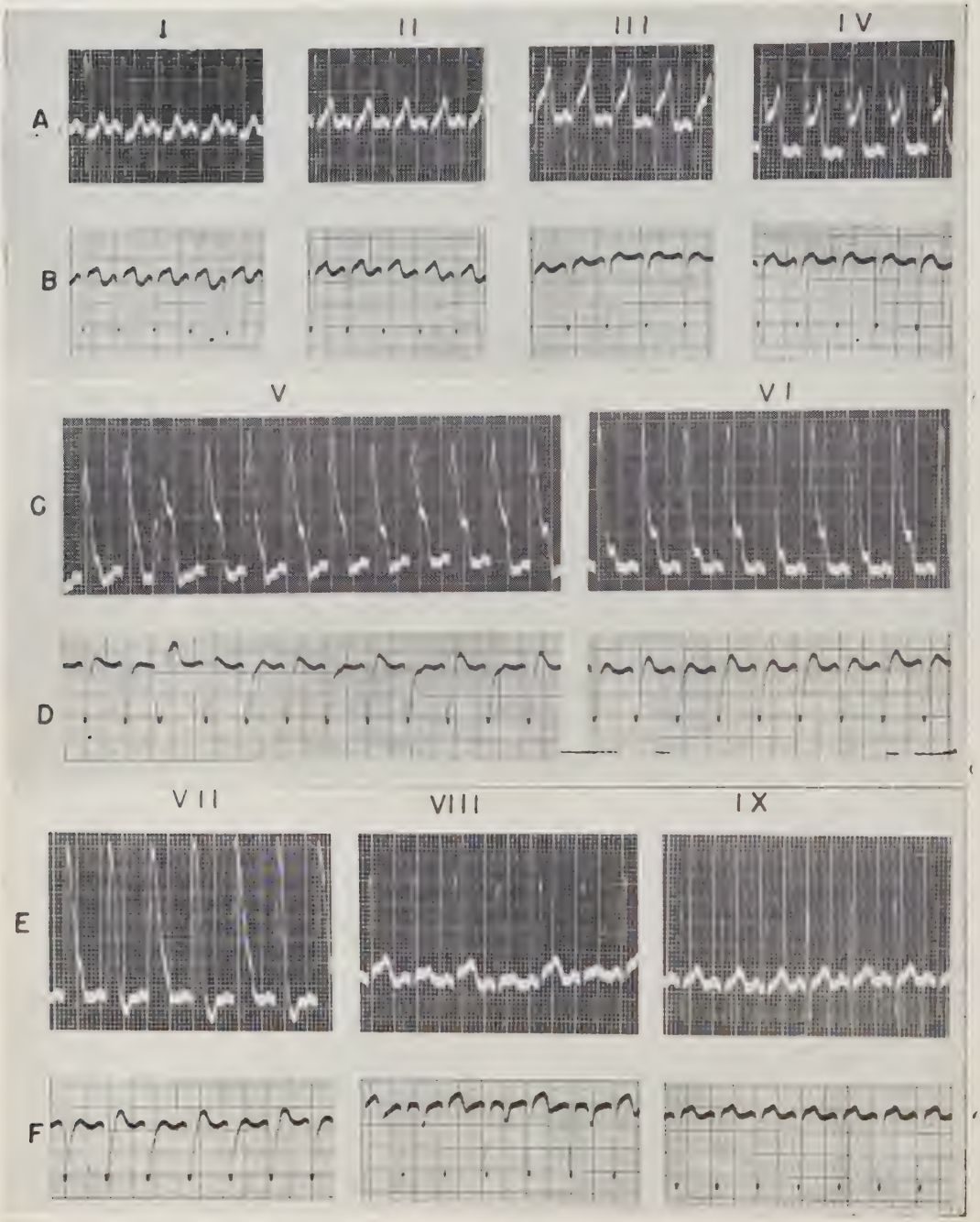

FIG. 1.

Electrical alternation following coronary artery ligation. Rows A, C, and E are epicardial records, and B, D, and F intracavitary records from left ventricle. Column 1 is control. Columns 2, 3, 4, 5, 6, 7, and 8 are 25, 55, 105, 243, 253, and 293 seconds after occlusion of left anterior descending artery. Note T wave changes precede ST deviation. Alternation is more marked after appearance of a premature ventricular beat (Column 5). ST-T alternation in Columns 5, 6, and 8. T wave alternates in direction in cavity lead in Column 8. Column 9 shows recovery 7 minutes after release of occlusion.

sion in intracavitary leads. Alternation of the QRS complex showed variation of less than 10% in QRS areas between large and small beats. Areas of the ST-T complexes varied on an average of 40% between beats.

Discussion. Fundamentally, the factor underlying all forms of alternation is a marked prolongation of the refractory phase of some part of the heart leading to alternating localized block.[3,4] In Wiggers' laboratory, Orias[5] and Tennant[6] showed that masses of cardiac fibers are deleted from contracting as a result of ischemia following coronary artery occlusion. Green[7] demonstrated that this deletion may occur alternately, *viz.*, mechanical alternation occurred after experimental coronary artery occlusion with the area of ischemia bulging alternately, and producing an alternation of aortic pressure. Similarly, we may assume that electrical alternation of injury effects seen in our experiments is on the same basis, *i.e.*, a failure of certain fractions of ischemic myocardium to respond on alternate beats.

Alternation was predominantly of the ST-T complex, probably because injury currents (of rest and of activity)[1] persist longer than the transient initial changes of depolarization and repolarization in early acute myocardial ischemia.[2] Alternation in the direction of the T wave implies alternation in the epicardial-endocardial order of repolarization, while alternation of the magnitude of the T wave implies alternation of the rate or intensity but not of the direction of repolarization.[8]

Previous temporary occlusions predisposed the myocardium to the production of alternation. The etiological significance of ventricular premature beats remains unsettled since alternation occurred with or without preceding ventricular premature beats. Heart rate was not significantly increased, so that electrical alternation cannot be explained on this basis.[9]

Summary. Electrical alternation developed in 8 of 9 dogs (89%) which survived coronary artery occlusion and showed electrocardiographic signs of myocardial ischemia. Electrical alternation occurred within 2 to 3 minutes after occlusion and was transient. Repeated temporary occlusions predisposed to the development of electrical alternation.

Alternation was predominantly of the ST-T complex, although less marked alternation of the QRS complex and of the T wave also occurred.

It is postulated that in our experiments, electrical alternation of injury effects is due to the failure of certain fractions of ischemic myocardium to respond on alternate beats.

The authors express their appreciation to Dr. Harold Feil for his helpful criticism and guidance.

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16858

Antituberculous Activity and Toxicity of Lupulon for the Mouse.*

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Wong and others have reported from the University of California on the *in vitro* activity of subtilin against *Mycobacterium tuberculosis*.[1-4] The parenteral application of this antibiotic has been interfered with by its relative insolubility in physiologic saline.[5] Antibiotics characterized by lipid solubility might overcome this difficulty. Two such agents prepared at the Western Regional Research Laboratory are lupulon and humulon derived from hops (*Humulus lupulus, L.*). Lupulon has the structure given below:[6]

In humulon the side chain marked "*" is replaced by a hydroxyl group. A method for the isolation of lupulon from hops by direct crystallization was discovered by Michener *et al.*[7] and further simplified by Alderton.[8] The initial crystallization occurred in a vacuum concentrate of a direct petroleum ether extract of hops.

The antibacterial properties of hops have been recognized for many years, in connection with their use in brewing. Walker and Parker[9] reported lupulon and humulon to be 29,000 and 4,000 times, respectively, as active as phenol in restricting acid production by *Lactobacillus bulgaricus*. Shimwell[10] noted that hop extracts inhibited a considerable number of saprophytic Gram-positive bacteria, but not Gram-negative bacteria. Acid-fast mycobacteria and other pathogens were not tested. Michener *et al.*[7] reported antifungal activity for lupulon and humulon. Subsequent to the earlier observations reported in this paper, Reynolds[11] found inhibition of acid-fast organisms at 1:100,000 for lupulon and at 1:10,000 or 1:20,000 for humulon when this agent was incorporated in agar by a streak technic. Salle[12] obtained similar results against *M. tuberculosis* tested on both Long's and Dubos' media.

Hops have long been regarded as having hypnotic properties. Steidle[13] reported that hops produce paralysis and decreased excitability of the striated muscle and of motor nerve endings in frogs. Sikorski and Rusiecki[14] reported lupulon and humulon to be sedative for pigeons and small birds and somewhat less active in mice. These indications of pharmacologic action after oral administration, together with the fat-soluble nature of

* University studies were supported in part by a grant from Eli Lilly and Company, Indianapolis 6, Indiana.

† Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

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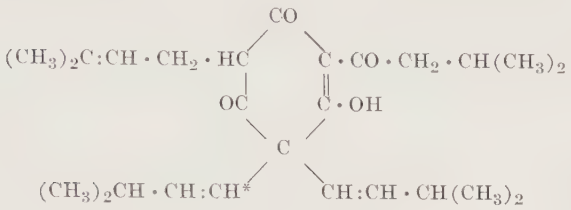

lupulon, made preliminary tests of the effect of this agent on experimental tuberculosis infections desirable.

Toxicity of lupulon. The toxicity of lupulon on single and repeated intramuscular injections was determined in inbred white mice. Crystals of lupulon were dissolved (1.5%) in cottonseed oil. The acute LD_{50} was found to be 600 mg per kilo. Deaths occurred within 1 to 12 hours. At autopsy, it was noted that incomplete absorption of the solution had occurred even after 5 days. This condition was observed in animals given only half of the LD_{50}. Daily injection of 60 mg per kilo over 4 weeks was tolerated without gross evidence of harmful effects. Histopathologic examination of tissue from these animals revealed small areas of leukocytic infiltration of the liver as well as foci of degeneration in the renal tubules.

Oral toxicity of lupulon, 5% suspended in gum acacia solution (6% in water) was determined in mice. Intragastrically, 1500 mg per kilo killed half of the animals within one hour of administration. All treated mice were depressed in contrast to those animals given the drug intramuscularly. Immediately before death, mice given doses twice the LD_{50} were excited and exhibited convulsions of a tetanic character. Congested and hemorrhagic lungs were observed at autopsy.

Antituberculous activity. Six samples of crystalline lupulon have been tested *in vitro* against tubercle bacilli (H37Rv) in Dubos' fluid medium.[15] Serial dilutions were made by pipetting appropriate amounts of lupulon solution in propylene glycol into 5 ml of the medium. Early samples were used in 1% solution in propylene glycol with the aid of heat, and later samples were dissolved first in 95% ethyl alcohol up to 10% solution and then diluted to 0.1% in propylene glycol. Growth of mycobacteria was inhibited at a dilution of 1:40,000 whether heat or ethyl alcohol was used to facilitate solution of the lupulon in the glycol. Humulon had a much lower potency and was not investigated further.

Various wetting agents,‡ including "Tween 60," "G-2144," "Nopacol," "Neutronyx," and "Triton," have been used to substitute for "Tween 80" in Dubos' medium without any effect on the tuberculostatic activity of lupulon.

The activity of lupulon has also been tested in serial dilution in the presence of a number of other antituberculous substances such as promin, streptomycin, subtilin, and 2,3-dimercaptopropanol (BAL). Sub-bacteriostatic concentrations of the latter agents (*e.g.*, 1:800, 1:3,200,000, 1:400,000 and 1:100,000, respectively) were used in the Dubos medium. Lupulon checked the growth of tubercle bacilli at 1:40,000 despite the presence of any of these agents. No synergism or antagonism occurred. The lack of synergism between lupulon and BAL is in contrast with the potentiation of action of subtilin and streptomycin by BAL, previously reported.[3]

Groups of 20 mice were infected intravenously with 0.02 mg of the H37Rv strain of *M. tuberculosis* grown in Dubos' medium. Lupulon was administered by two different routes. In one group of infected animals, it was given intramuscularly as a 1.5% solution in cottonseed oil at a daily single dose of 60 mg per kilo. In another group, it was administered intragastrically as a 3% suspension in 6% gum acacia solution (aqueous)

[15] Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

‡ Kindly supplied by Dr. R. J. Dubos, Rockefeller Institute for Medical Research, New York. Mention of this and other products does not imply that they are endorsed or recommended over others of a similar nature not mentioned.

TABLE I.
Tuberculostatic Activity of Lupulon in Infected Mice.

Antibiotic	Experiment	Route	Approx. LD_{50} mg/kg	Daily dose mg/kg over 30 days	Local effects noted grossly	Avg gross lung lesions*	Tissue reaction in all organs examined	Relative abundance of T.B. in all organs†	No. animals in each group
Controls	I	—	—	—	—	3.7	18	713	20
,,	II	—	—	—	—	5.0	24	1,514	20
Lupulon	II	Oral	1500	150	None	2.3	18	382	20
,,	I	I.M.	600	60 (twice daily)	,,	1.9	12	231	20
,,	II	I.M.	600	60 (daily)	,,	2.8	16	368	20

* *Tissue Reaction:* Number indicates total (average) degree of lesions (larger means greater involvement); *e.g.*, Lung: 8 = 50-100% of tuberculous tissue in the whole organ as observed grossly as well as microscopically; 4 = 25-50%; 2 = 10-25%; 1 = 1-10%; 0 = no gross lesions. Spleen and liver: 8 = 10 lesions per low power field; 4 = 5-10 lesions; 2 = 2-5; 1 = lesions in any portion; 0 = no apparent lesions. Kidney and heart: Number = No. of lesions in the section; exception 4 = 4 or more.

† *Abundance of Mycobacteria:* Number indicates total (average) relative number of mycobacteria in all lesions; *e.g.*, 1,000 = more than 10 bacilli to each cell; 100 = 1-10 to each cell; 10 = 1 bacillus to 1-10 cells; 1 = 1 bacillus to 1-5 lesions; 0 = no organisms.

at a dose of 150 mg per kilo at 12-hour intervals. Other infected animals served as untreated controls. The results are shown in Table I. The evaluation of the gross tuberculous lesions in the organs was based on histopathologic sections stained with hematoxylin and eosin for lesions and by acid-fast methods for determining abundance of tubercle bacilli present.

Despite its relatively low *in vitro* activity, lupulon exerted considerable suppressive effect on the development of tuberculosis in mice, whether it was given intramuscularly or orally. It appeared to check the multiplication of tubercle bacilli to a similar extent when given orally. The latter method gave greater suppression in the development of tuberculosis in the second series of experiments where a difference in multiplication of mycobacteria was apparent.

Table II gives further details regarding the relative abundance of mycobacteria in lesions of treated and untreated mice. In comparing the prevalence of microorganisms in the lungs, spleen, liver, kidneys, and heart of the control animals, notable differences were apparent at the end of the 30-day period with their relative abundance in lupulon-treated animals. The ratios (in Experiment I) approximated from 34 to 1 (comparison of controls with lupulon-injected mice) for liver, 8 to 1 for heart, 4 to 1 for spleen, and 4 to 1 for lungs. After oral use (in Experiment II) the ratios were: Liver 10 to 1, heart 3 to 1, spleen 10 to 1, lungs 5 to 1, kidneys 2 to 1: an over-all 4 to 1 difference. Only the renal lesions had a slight and perhaps insignificantly greater number of mycobacteria in lupulon-injected animals, in the first but not in the second series. Renal damage might be related to tubular changes which followed continual administration of this antibiotic, and these may have been accentuated by the extent of renal infection, with concurrent drug damage to this tissue. Another possibility is that the foci of degeneration noted in the renal tubules (in infected intramuscularly treated animals) may have permitted more extensive tuberculous involvement.

The lupulon-treated mice had lower indices

TABLE II.
Relative Abundance of Mycobacteria* in Lesions of Treated and Untreated Mice.

Antibiotic	Experiment	Route	Lungs	Spleen	Liver	Kidneys	Heart	Sum total
Controls	I	—	471	19.3	68.3	70.7	83.8	713
,,	II	—	353	277	278	170	436	1514
Lupulon	II	Oral	78	27	27	80	170	382
,,	I	I.M.	121	4.5	2.0	93.4	10.1	231
,,	II	I.M.	14.6	3.8	12.9	2.7	334	368

* See Table I for key.

of tissue reaction, as measured by the development of lesions, in all but the renal and cardiac tissues. The latter organs in all groups did not differ appreciably in occurrence of lesions. In the lungs, not only was there a lower percentage of tuberculous tissue in lupulon-treated mice than in the controls but the type of tissue reaction was also different. The lesions in the lungs in the lupulon-treated mice were predominantly proliferative, while those in the controls were predominantly necrotic and exudative.

Discussion. An over-all examination of these data showed a significantly lower number of mycobacteria in lesions of lupulon-treated mice. Thus far, in chemotherapeutic trials in animals, only indefinite numbers of mycobacteria in lesions have been reported.[16–18] In our opinion, the numbers of mycobacteria in the lesions is of great importance to the solution of the problem of ultimate control. The use of a drug should be for a relatively short period in terms of the life span of the individual. On cessation of therapy, there should be left in lesions a small enough number of bacilli for the natural defense mechanism of the host to combat successfully.

Since the lipid fraction of tubercle bacilli is a major part of the organism, it would appear reasonable to expect a fat-soluble antibiotic to have a marked affinity for these bacteria, and that this affinity might be reflected in the animal. Fat-soluble usnic acid, which has a relatively low tuberculostatic effect *in vitro,* has a definite effect in guinea pigs.[19] This hypothesis is also suggested by our observations; *e.g.,* the lipid soluble lupulon is active *in vivo.*

Lupulon, like the aerosporins and polymyxins, appears to have an affinity for the renal tubules when it is given intramuscularly. Whether these changes in the tubules are reversible or not remains for further experiment. The relatively mild leukocytic infiltration of the liver may not be significantly harmful. This may indicate a mobilization of the antibiotic from the site of injection and its storage in the liver. Storage in the liver would be compatible with the finding that the greatest reduction in mycobacteria occurred, among lupulon-treated animals, in this organ.

Summary. Lupulon, a fat-soluble antibiotic derived from hops, has relatively low *in vitro* activity (1:40,000) as compared with other antituberculous agents. Despite this observation, lupulon (given orally or intramuscularly) *was active* against experimental mouse infections of *M. tuberculosis.* Following intramuscular administration, significantly lower numbers of acid-fast organisms occurred in lesions of treated animals. The approximate numbers relative to the control were: In the liver, 34 to 1; heart, 8 to 1; spleen, 4 to 1; and lungs, 4 to 1; but not in the kidneys. In orally treated animals the ratios were: Liver, 10 to 1; heart, 3 to 1; spleen, 10 to 1; lungs, 5 to 1, and kidneys, 2 to 1. The over-all difference was a reduction of approximately 4 to 1 by either route of administration.

Lupulon given intramuscularly within its effective range produced some foci of degen-

16 Pierce, C., Dubos, R. J., and Middlebrook, G., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 173.

17 Youmans, G. P., Raleigh, G. W., and Youmans, A. S., *J. Bact.,* 1947, **54**, 409.

18 Levaditi, C., and Vaisman, A., *Comp. rend. Soc. Biol.,* 1948, **142**, 43; *ibid.,* 1948, **142**, 308.

19 Marshak, A., *Public Health Reports,* 1947, **62**, 3.

eration in renal tubules. Such pathologic changes were not observed in infected animals given this antibiotic orally in effective doses. The single LD_{50} on intramuscular administration was 600 mg per kilo in mice; on oral application, 1,500 mg per kilo.

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Tocopherol vs. Tocopherol Acetate as a "Sparer" of Vitamin A.

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The early observation[1] that materials containing vitamin E delayed the auto-oxidative destruction of carotene and vitamin A in fatty food mixtures was explained when the tocopherols were shown to be antioxidants.[2] That such protection may augment and conserve the vitamin A stores of an animal was first demonstrated by Moore[3] and has been confirmed repeatedly, as detailed in several reviews and some recent reports.[4]

The evidence suggests that inhibited oxidation in or near the alimentary tract[5] accounts for the survival of carotene and vitamin A. Recently,[6] this protective action appeared to be confined to preserving the store of vitamin A already deposited in the liver. The various forms of vitamin A behave differently. The results are modified by the form and method of administering the preparations of vitamin A[7] and vitamin E[8]. Other compounds having possible stabilizing effects[9] may be present and effective.

Several reports have stated or implied that the stabilizing action of the tocopherols is not limited to the alimentary tract but extends to the tissues of rats.[8,10] This was not demonstrable in rabbits.[11] Some information on this question might emerge from experiments with the esters or α-tocopherol, among them the acetate, which is also an oil, and is a dependable source of vitamin E, perhaps because it is not auto-oxidizable and has no antioxygenic action. Presumably, α-tocopherol acetate is hydrolyzed in the intestinal tract, and the free alcohol should therefore be available in the tissues in quantities undiminished by having provided prior stabilization in the food.

The only pertinent observation on the acetate appears to be that of Bacharach,[12] to the effect that only at high levels of feeding and for an extended period (60 days) was any conservation of vitamin A demonstrable. A comparison of α-tocopherol and its acetate was therefore undertaken, to assess the relative protective value of each by simple experiments patterned after the U.S.P. bio-

1 Mattill, H. A., *J. Am. Med. Assn.*, 1927, **89**, 1505.

2 Olcott, H. S., and Emerson, O. H., *J. Am. Chem. Soc.*, 1937, **59**, 1008.

3 Moore, T., *Biochem. J.*, 1940, **34**, 1321.

4 Hickman, K., *Ann. Rev. Biochem.*, 1943, **12**, 353; Moore, T., *Vitamins and Hormones*, 1945, **3**, 1; Mattill, H. A., *Ann. Rev. Biochem.*, 1947, **16**, 177; McCoord, A. B., *et al.*, Food Technol., 1947, **1**, 263; Foy, J. R., and Morgareidge, K., *Analyt. Chem.*, 1948, **20**, 304.

5 Hickman, K., *et al.*, *J. Biol. Chem.*, 1944, **152**, 303, 313, 321.

6 Popper, H., Steigmann, F., and Dyniewicz, H. A., *Gastroenterology*, 1948, **10**, 987.

7 Halpern, G. R., and Biely, J., *J. Biol. Chem.*, 1948, **174**, 817.

8 Lemley, J. M., *et al.*, *J. Nutrition*, 1947, **34**, 205.

9 Sherman, W. C., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 207; Esh, G. C., and Sutton, T. S., *J. Nutrition*, 1948, **36**, 391.

10 Davies, A. W., and Moore, T., *Nature*, 1941, **147**, 194; Hove, E. L., and Harris, P. L., *J. Nutrition*, 1946, **31**, 699; Lundberg, W. O., *et al.*, *J. Biol. Chem.*, 1947, **168**, 379.

11 Major, R., and Watts, B. M., *J. Nutrition*, 1948, **35**, 103.

12 Bacharach, A. L., *Quart. J. Pharm. Pharm.*, 1940, **13**, 138.

assay for vitamin A.

Experimental. d,l,α-tocopherol acetate. Rats of the Sprague-Dawley strain were given a stock diet until they weighed 40-50 g. Half of them were then transferred to a diet deficient in both vitamins A and E and having the following percentual composition: Vitamin-free casein, 20%; glucose, 67.5%; salt mixture,[13] 2.5%; ground cellophane, 3%; lard, 2%; yeast, 5%; viosterol (in peanut oil), 40 drops per kg. The other half were given the same diet except that hydrogenated vegetable fat (Spry) replaced the lard and 12.5 μg of *d,l,α*-tocopherol acetate* were added per kg of diet. Based on food consumption each animal received 70-80 γ of α-tocopherol acetate and 4-5 γ of tocopherols daily. In a second series the amount of α-tocopherol acetate added to the diet was 100 mg per kg, giving a daily intake of about 600 γ.

At the end of the deprivation period of 3-4 weeks when xerophthalmia or decline in weight or both became evident, the animals were properly grouped and vitamin A containing oils were administered daily, orally, by syringe with bent blunt needle. The Standard U.S.P. Reference Oil No. 2 and the several shark liver oils† to be tested were diluted with cottonseed oil‡ to contain 2 I.U. of vitamin A per 0.1 cc (Reference Oil) or per 0.05 cc (liver oils). The oils were kept in the cold and new dilutions were made up fresh each week.

In Table I are the average figures obtained from these two series of animals. They indicate surprising and certainly acceptable uniformity in the times required for the development and cure of the symptoms of deficiency, whether α-tocopherol acetate was present in the diet or not. The weight gains on the reference oil were only half as great as those on the test oils, and the cure of xerophthalmia was also slower. This was doubtless due to the fact that this oil, as was reported later,[14] contained 30% less vitamin A than indicated. This figure was subsequently verified by the Carr-Price method against a standard vitamin A capsule kindly supplied by Distillation Products, Inc.

The vitamin A potency of the fish oils as indicated by the anti-xerophthalmic data agreed well with that of the chemical assays provided (Carr-Price, Rosenthal, Beckman). With the figures for gain in weight, the agreement was unsatisfactory, largely because of the unsuspected inferiority of the reference oil, and the consequent lack of a comparable standard dose-level.

d,l,α-tocopherol. The tests with the alcohol, made some time after those just described, differed from the earlier ones in several minor respects. Drisdol was used in place of viosterol as a source of vitamin D. Neither α-tocopherol nor vegetable oil was added to the basal diet of the experimental animals; α-tocopherol§ was given to them by mouth during the assay period only and in the same diluting olive oil which also contained the 2 I.U. of the vitamin A. The control animals received their vitamin A in olive oil. The sources of vitamin A were limited to the two reference oils U.S.P. Nos. 2 and 3 (the former after several years' storage in a dark ice-box). Finally, in lieu of a chemical determination of the liver stores of vitamin A, which at the moment could not be made on the necessary micro-scale, the survival time was determined after the close of the assay

[13] Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, 1937, **14**, 273.

*Generously supplied by Hoffman-La Roche, Inc.

† We are indebted to Dr. J. Murray Luck for samples of these oils and the results of chemical assays on them, on the basis of which they were diluted for the bio-assays.

‡ Cottonseed oil was used designedly, if not wisely, to stabilize the vitamin A in the oils before absorption. During the assay period, therefore, the vitamin E deficient animals on the test and reference oils received daily 20 and 40 γ of tocopherols, respectively; those on the low level of dietary α-tocopherol acetate had a 20-50% increase in the potential vitamin E intake, those on the high level, 3-7% increase. The data indicate that cottonseed oil was inconsequential in its effect on the final results and that it may have accomplished its purpose.

[14] Collison, E. C., and Orent-Keiles, E., *Ind. Eng. Chem.*, Anal Ed., 1945, **17**, 378.

§ Kindly supplied by Merck and Company.

TABLE I.
Influence of α-Tocopherol Acetate on Bio-assay of Vit. A.
+E animals had 12.5 mg *d,l*,α-tocopherol acetate/kg of diet.

Source of curative Vit. A		Avg body wts at depletion, g	Avg gain in assay period, g	Xerophthalmia: Appeared in depletion period at avg days	Xerophthalmia: Cured in assay period at avg days
Refs. Oil 2	—E	84 (6)*	28 (1)†	23	22
Refs. Oil 2	+E	79 (6)	27 (1)	22	20
Oil 3	—E	74 (5)	58 (2)	23	21
Oil 3	+E	80 (8)	52	23	16
Oil 4	—E	80 (8)	46	24	18
Oil 4	+E	80 (7)	46	23	16
Oil 5	—E	80 (8)	47	23	19
Oil 5	+E	82 (7)	44 (1)	23	16
	+E animals had 100 mg *d,l*,α-Tocopherol acetate/kg of diet.				
Refs. Oil 2	—E	76 (6)	25 (1)	28	17
Refs. Oil 2	+E	78 (6)	33 (1)	22	18
Oil 7	—E	76 (8)	65	23	11
Oil 7	+E	74 (7)	54	22	15
Oil 11	—E	75 (5)	50 (3)	25	10
Oil 11	+E	76 (7)	54	23	11
Oil 14c	—E	72 (6)	57 (1)	25	10
Oil 14c	+E	72 (7)	53 (1)	22	12

* No. of animals.
† No. of animals which died during the period and not included in the average.

TABLE II.
Influence of α-Tocopherol on Bio-assay of Vitamin A.

Reference oil and amt. of tocopherol, (mg)	Avg body wt at depletion, g	Gain in assay period, g	Xerophthalmia: Appeared in depletion period at avg days	Xerophthalmia: Cured in assay period at avg days	Avg survival in post-assay depletion, days
2 .0	85 (8)*	3	29	13	11
2 .5	91 (6)	17 (2)†	31	12	19
2 1.5	91 (6)	11 (2)	29	13	19
3 .0	90 (5)	4 (4)	30	16	14
3 .5	80 (8)	25	28	15	19
3 1.5	87 (7)	13 (2)	34	10	19
2 .0	80 (7)	11 (1)	27	11	17
2 .3	85 (5)	24 (3)	28	6	24
3 .0	82 (8)	24	27	7	15
3 .7	78 (7)	38 (1)	27	8	28

* No. of animals.
† No. of animals that died during the assay period and not included in the average.

period when the administration of supplements ceased. Two series of observations were made with each of the reference oils; in the first, 0.5 and 1.5 mg of α-tocopherol were administered with each oil, in the second 0.3 mg with oil No. 2, and 0.7 mg with oil No. 3.

Despite the unexplained high mortality in some of the lots, the data in Table II show that (1) survival in the post-assay depletion period was prolonged by α-tocopherol given in the assay period; (2) there was an optimum α-tocopherol dosage, perhaps 0.5-0.7 mg daily, beyond which the effectiveness of the vitamin A dosage decreased, as indicated by gains in weight in the assay period. Both of these findings are confirmatory of earlier reports[5] and point to an antioxygenic rather than a biological function of α-tocopherol. The stabilization of fats by α-tocopherol *in vitro* also demonstrates an optimum concen-

tration; in the case of lard, this is between 0.05 and 0.10% of α-tocopherol.[15] Very recently, in similar experiments,[16] there is a suggestion that 0.5 mg α-tocopherol is an optimum dosage for the storage of vitamin A in the liver; the effect on vitamin A storage in the kidney was irregular. The liver stores of vitamin A from administered β-carotene were diminished by the larger doses of α-tocopherol and kidney storage was again irregular.

Unfortunately, no indication was secured as to the vitamin A stores of our animals on α-tocopherol acetate, following the assay period. The larger of the two dosages, 0.6 mg per day, even though it was within the optimum range and extended over both depletion and assay periods, may have been insufficient; it can hardly have been excessive. In any case, there was no response. The stabilization which the acetate can provide for vitamin A in the alimentary tract is limited to the amount of α-tocopherol available from it in the interval between hydrolysis and absorption. Perhaps, as with triglycerides, complete hydrolysis of the ester is not necessary for absorption, in which case any protective action in the tissues would depend upon the amount absorbed and the rate of subsequent hydrolysis. Information is needed on the rate of hydrolysis and absorption of tocopherol esters. The possible antioxygenic role of tocopherols in the tissues remains to be clarified.

Summary. 1. In bio-assays of vitamin A, α-tocopherol, but not α-tocopherol acetate, increased the rate of gain in the assay period. In a post-assay depletion period, animals that received α-tocopherol during the assay period survived 30-80% longer than controls.

2. As judged by gain in weight in the assay period, there is an optimum dosage of α-tocopherol; animals receiving 1.5 mg per day gained less than those receiving 0.5 mg. This conforms to the antioxygenic behavior of α-tocopherol.

3. Some of the possible causes for the failure of α-tocopherol acetate to conserve vitamin A are briefly discussed.

[15] Golumbic, Calvin, *Oil and Soap*, 1943, **20**, 105.

[16] Johnson, R. M., and Baumann, C. A., *J. Biol. Chem.*, 1948, **175**, 811.

16860 P

On the Specificity and Differentiation of Cholinesterases.

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Cholinesterases (ChE) of different sources may be separated into two types, the "s" and the "e."[1-3] The behavior of the two enzymes toward noncholine esters has been supposed to be the major difference between them. It has been assumed that the e-type could catalyze only the hydrolysis of certain choline esters;[4] therefore it has been also called "specific" or "true" cholinesterase.

The venoms of the colubrid snakes can split acetylcholine[5] and some noncholine esters.[6] In a recent investigation, it was shown that

* On leave from the University of Basel, Switzerland.

[1] Richter, Derek, and Croft, Phyllis G., *Biochem. J.*, 1942, **36**, 746.

[2] Mendel, B., and Rudney, H., *Biochem. J.*, 1943, **37**, 59.

[3] Zeller, E. A., and Bissegger, Alfred, *Helvet. chim. acta*, 1943, **26**, 1619.

[4] Mendel, B., Mundell, Dorothy B., and Rudney, H., *Biochem. J.*, 1943, **37**, 473.

[5] Zeller, E. A., Enzymes of Snake Venoms and Their Biological Significance. In: *Advances in Enzymology and Related Subjects of Biochemistry* (edited by F. F. Nord), New York, Interscience Publishers, Inc., 1948, **8**, 459.

[6] Bovet Nitti, F., *Experientia*, 1947, **3**, 283.

several noncholine esters—for example, ethyl chloroacetate (EC1A)—are hydrolyzed at a high rate by many of the colubrid venoms, and that this hydrolysis is due to cholinesterase.[7] Since the colubrid cholinesterase belongs to the e-type,[8] the question arose as to whether other e-cholinesterases also have the ability to split EC1A.

In order to solve the problem of whether other cholinesterases display a pattern of specificity similar to that of the snake venom enzyme, experiments were carried out with the e-cholinesterase of the erythrocytes of human beings. The hemolyzed erythrocytes and a purified preparation derived therefrom[2] catalyzed the hydrolysis of EC1A at an easily measurable rate. With the use of mixtures of acetylcholine and EC1A, it could be shown that with the concentration of acetylcholine varying from 0.004 M to 0.025 M, and with the concentration of EC1A varying from 0.035 M to 0.075 M, the resulting hydrolysis rate ($Q_{ACh + EC1A}$). One typical example is given in Fig. 1.

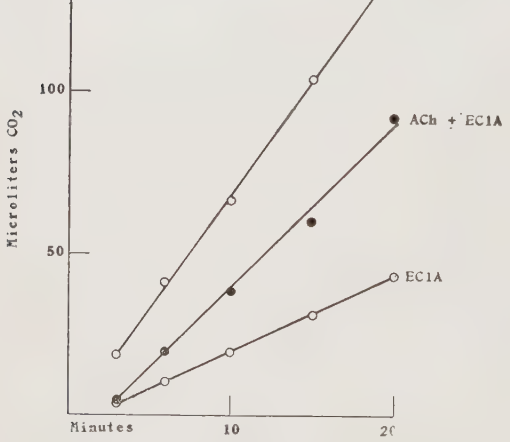

FIG. 1.

Hydrolysis of ethyl chloroacetate and acetylcholine by hemolyzed erythrocytes. Manometric measurement of the CO_2 produced from bicarbonate by the liberation of acids.[9] Erythrocytes were washed three times with saline, hemolyzed with an equal volume of distilled water and diluted with two volumes of bicarbonate-*Ringer* ("Ringer-30"). Three tenths milliliter of this preparation was used in a total volume of 3 ml. Final concentration of acetylcholine: 0.0067 M; final concentration of EC1A: 0.075 M. Blanks resulting from the enzyme solutions and the substrates were subtracted.

TABLE I.
Inhibition of the Enzymatic Hydrolysis of EC1A by Eserine.

Source of cholinesterase	Type	Inhibition, %
Erythrocytes (human) purified*	e	84-94
Snake venom (Naia melanoleuca)†	e	100
Plasma (human)‡	s	4-11
Parotid (guinea pig)§	s	13-

Same method as described in Fig. 1.

* 0.2 ml of erythrocyte-ChE, prepared according to the method of Mendel and co-worker.

† 40 μg of dried venom of Naia melanoleuca.[10]

‡ 0.083 ml of heparinized plasma of human beings.

§ 0.5 cc of extract from homogenized parotids from guinea pigs, prepared with 10 volumes of saline solution. Final concentration of eserine: 0.2-0.3 10^{-3}; final concentration of EC1A 0.016-0.05 M.

The enzymatic hydrolysis of EC1A in the presence of snake venoms[10] and of purified ChE from human erythrocytes[2] is inhibited by caffeine and other hydroxylpurines and by eserine (Table I) and prostigmine in the same way, and to the same extent, as has been shown to be true of acetylcholine.[3,11]

Serum from human beings and extracts from guinea pig parotid glands, typical sources of the s-ChE ("unspecific," "pseudo" ChE), split EC1A very rapidly. This hydrolysis is only slightly inhibited by eserine (Table I).

Another striking difference between the two esterases was found when β-chloroethyl acetate was used. This noncholine ester is easily attacked by purified erythrocytes and snake venom. Experiments carried out with mixed substrates and with eserine gave the same results as with EC1A, indicating that this substance is also hydrolyzed by e-ChE. Human serum is comparatively less able to

7 Zeller, E. A., *Helvet. physiol. et pharmacol. acta*, 1948, **6**, C36.

8 Zeller, E. A., *Experientia*, 1947, **3**, 375.

9 Ammon, R., *Arch. f. d. ges. Physiol.*, 1933, **233**, 486.

10 Zeller, E. A., and Utz, D. C., *Helvet. chim. acta*, in press.

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TABLE II.

Enzymatic Hydrolysis of β-Chloroethyl Acetate and Acetylcholine.

Same method as described in Fig. 1; same enzyme preparations as in Table I. Final concentration of acetylcholine: 0.0067 M; final concentration of β-chloroethyl acetate; 0.033 M for the first three and 0.016 M for the last preparation. Q_{ACh} resp. $Q\beta_{C1A}$ give the number of microliter of CO_2 produced in 1 hour, calculated from reaction of zero order.

Source of cholinesterase	Type	Q_{ACh}	$Q\beta_{C1A}$
Erythrocytes (human) purified	e	669	249
Snake venom (Naia melanoleuca)	e	540	129
Plasma (human)	s	405	39
Parotid (guinea pig)	s	711	72

split this ester (Table II).

Detailed information about methods and enzyme and substrate preparations will be given in an extensive publication.

Conclusions. The ability to hydrolyze noncholine esters can no longer be used as a criterion to distinguish the two cholinesterase types, since both groups of enzymes are able to split noncholine esters; for example, ethyl chloroacetate. Another noncholine ester, β-chloroethyl acetate, is even preferentially attacked by the e-type ("true" or "specific" ChE).

The use of eserine to decide whether a hydrolysis is catalyzed by a cholinesterase or another esterase is of limited value, because the s-type ("pseudo" or "unspecific" ChE) is inhibited by this substance only in the presence of acetylcholine, but not in that of ethyl chloroacetate. The e-type is inhibited in both cases.

Ethyl chloroacetate plus eserine and β-chloroethyl acetate can be used as new means of distinction of the two groups of cholinesterases.

16861 P

Variations in the Vitamin B_{12} Content of Selected Samples of Pork and Beef Muscle.*

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In a recent paper[1] a fairly quantitative assay procedure for a growth factor in liver preparations was described. This assay procedure involves placing rats on the basal ration for a 2 week depletion period and following the growth response during another 2 week period when the material to be tested is given. Further work has shown that crystalline vitamin B_{12} will give a quantitative response in this assay (Fig. 1).

Henderson *et al.*[2] have reported wide variations in the ability of pork muscle, when fed to female rats as the source of protein, to produce normal lactation. Less than one-third of the young were reared in certain experiments; however, in other experiments the lactation was comparable to that obtained with rations containing beef. Beef-fed rats

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

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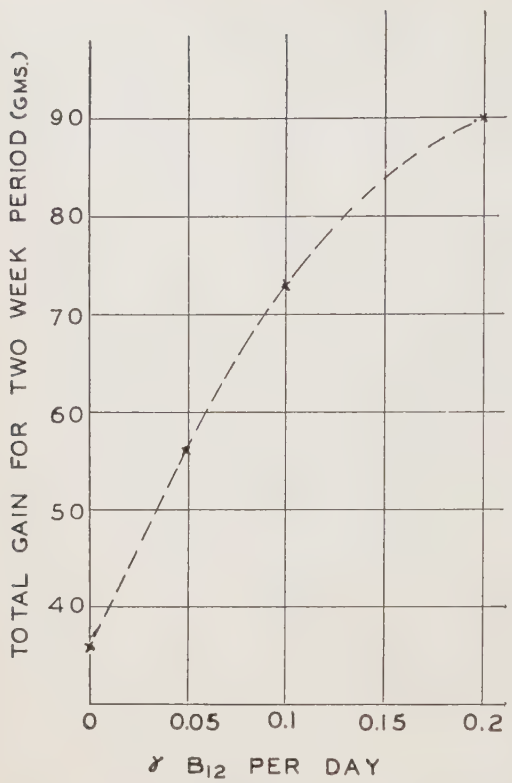

Fig. 1.
Response of rats receiving graded levels of B_{12}.

usually reared 80 to 90% of the young. The wide variations encountered when pork was fed as the source of protein led to the testing of samples of beef and pork by this new assay method for B_{12} activity.

A commercial liver preparation, assayed for vitamin B_{12} content by means of rats using crystalline vitamin B_{12} as a standard, was used as a reference. One USP antipernicious anemia unit of liver preparation was found to contain approximately one microgram of vitamin B_{12}.

These results show that the beef samples contain twice as much B_{12} as the normal pork sample while the abnormal pork sample contains only a trace or no B_{12}. The results given for the meat samples are minimum values since they are compared with B_{12} which was injected intraperitoneally. The wide variation in the B_{12} content of pork samples is significant in that it may offer an explanation for the variations in the ability of pork samples to produce normal lactation in female rats reported by Henderson and coworkers.[2]

The variable results with pork are not surprising since swine, being monogastric animals, may not be supplied with ample quantities of B_{12} and possibly other factors which may be produced by the microorganisms in the rumen of cattle.

We are indebted to Merck and Co., Inc., Rahway, N. J., for crystalline vitamins including B_{12}, and to Dr. B. L. Hutchings of the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., for synthetic folic acid.

Daily supplement	Total avg gain (g) two wk	Min. B_{12} (γ) per 100 g of sample
None	36	—
5 g beef, round steak (Sample 1)	74	2
5 g beef, round steak (Sample 2)	74	2
5 g pork, shoulder (normal)*	56	1
5 g pork, shoulder (abnormal)†	39	Trace
0.1 USP. unit Reticulogen (Lilly)‡	74	2000§

* From sow that lactated normally.
† From sow that showed abnormal lactation (young died after birth).
‡ Injected intraperitoneally.
§ Contains approximately 1 γ B_{12} per USP unit.

16862

Influence of Adrenergic Blocking Drug [N-Ethyl-N-(2-bromoethyl)-1-naphthylenemethylamine · HBr] On Pyrogenic Reaction.*

J. A. Wells and David P. Rall.

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An important mechanism of the febrile response to pyrogen is vasoconstriction[1-3] with reduction of skin temperature.[2,4-10] The site of action of pyrogen in the production of fever is central rather than peripheral since such fever is prevented by certain lesions of the central nervous system.[11-13] Pyrogen induced vasoconstriction and fever are mediated in part *via* the sympathetic nervous system, partial or complete removal of which modifies or prevents such vasoconstriction[2,5] and delays and reduces fever.[14] Evidence is here presented that N-Ethyl-N-(2-bromoethyl)-1-naphthylenemethylamine · HBr,† a potent adrenergic blocking agent,[15-17] reduces the febrile response of the dog to pyrogenic material.

Five dogs received intravenous injections of 50 mcg/kg of "Pyromen,"‡ a purified pyrogenic material obtained from pseudomonas aeruginosa. Six dogs were injected intravenously with 3 mg/kg of N-Ethyl-N-(2-bromoethyl)-1-naphthylenemethylamine · HBr 30 minutes prior to the injection of pyrogen. Five days later the order was reversed and dogs previously pre-treated with blocking agent now served as their own controls and vice versa.

Rectal temperatures were determined at 30 minute intervals and data from the experiments on these 11 dogs are given in Table I. The first value is the average temperature during the 6 hour period following injection of pyrogen, and the second is the maximum temperature recorded during the pyrogenic reaction. Both are expressed as increments over basal temperature. Paired comparisons show the mean responses of the two experimental periods to be significantly different. The reduction in average response accomplished by the blocking agent is of the order of 25%.

These studies imply only slight participation of the sympathetic nervous system in the pyrogenic reaction. However, while the dose of N-Ethyl-N-(2-bromoethyl)-1-naphthylenemethylamine · HBr is sufficient to cause complete reversal of the blood pressure response to injected epinephrine in the dog, its potency in preventing sympathetic nerve mediated

* Aided by a grant from Baxter Laboratories, Inc., Morton Grove, Ill.

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† Supplied by Parke, Davis and Co., through the courtesy of Dr. George Rieveschl, Jr.

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‡ Supplied by Baxter Laboratories, Inc., through the courtesy of Dr. N. M. Nesset.

TABLE I.
Effect of Adrenergic Blocking Agent on Pyrogenic Response in Normal Dogs.

Dog No.	Avg temp. increment over 6 hr (°C)		Maximum increment in temp. (°C)	
	Control	Pre-treated	Control	Pre-treated
1	1.50	1.33	2.05	1.94
2	0.89	0.83	1.33	1.22
3	1.39	0.61	1.72	1.11
4	1.11	1.05	1.67	1.50
5	2.05	1.05	2.55	1.61
6	1.72	1.33	2.10	1.78
7	1.83	1.33	2.44	1.89
8	1.45	1.22	2.50	1.61
9	1.56	1.28	2.16	1.72
10	1.55	1.33	2.22	1.89
11	1.11	0.67	2.11	1.16
Avg	1.469	1.094	2.077	1.585
Probability	<0.01		<0.01	
Significance	High		High	

All temperatures are rectal and are expressed as increments over basal temperature determined during pre-pyrogen control period.

vasoconstriction in the skin is unknown.

In order to further test the role of the sympathetic nervous system in the pyrogenic reaction, the effect of the adrenergic blocking agent on the pyrogenic reaction was determined in curarized dogs. Under such conditions the major portion of the fever following injection of pyrogen is due to reduced heat loss.[18] Since the curarized dog cannot reduce heat loss by reduction of ventilation or reduction of radiating surface, it may be assumed that vasoconstriction is the mechanism of the fever.

Crystalline d-tubocurarine§ was given intravenously in an initial dose of 1.5 mg/kg followed by hourly intramuscular injections of 0.5 mg/kg. Artificial respiration by means of a Starling-Palmer pump was adjusted to give a ventilation-oxygen consumption ratio of approximately 20. Rectal temperatures were obtained by means of a recording resistance thermometer. Room temperature was maintained at 27 ± 1°C.

Fifteen minutes after curarization 10 dogs were given 3 mg/kg of N-Ethyl-N-(2-bromoethyl)-1-naphthylenemethylamine · HBr by slow intravenous infusion. After a control period of approximately 1 hour these 10 dogs and 13 curarized control dogs were given 50 mcg/kg of pyrogen intravenously.

Data for the 13 control and 10 pre-treated animals are shown in Table II. Several values representative of the pyrogenic reaction are given. The first is the latent period or the period from injection of pyrogen to onset of fever. One effect of the adrenergic blocking agent appears to be significant prolongation of this latent period. The duration of the period of rising temperature is not significantly different in the two groups of dogs. The third value, the maximum increment in temperature during the pyrogenic reaction, is significantly lower in the group of dogs pre-treated with blocking agent. The mean response for the group is 52% less than that of the control. The fourth value, the average increment in temperature during the period of rising temperature, is significantly lower in the group of animals pre-treated with the blocking agent. The mean response of the pre-treated group of animals is 55% less than that of the controls.

Thus the adrenergic blocking drug produces 2 alterations of the pyrogenic reaction in curarized dogs. It delays the onset of the response and reduces its magnitude. The last entry in Table II is a single figure which portrays both of these processes. This figure is the average increment in temperature during the 2 hour period following the injection of pyrogen, and it is significantly lower in the

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§ Supplied by Abbott Laboratories, through the courtesy of Dr. R. K. Richards.

TABLE II.
Pyrogenic Reaction in 10 Curarized (Control) and 13 Curarized Dogs (Pre-treated) with Adrenergic Blocking Agent.

Latent period (min.)		Duration of rising temp. (min.)		Max. temp. post-inj. (°C)		Avg temp. during period of rising temp. (°C)		Avg temp. during 2 hr post-inj. (°C)	
Control	Pre-treated	Control	Pre-treated	Control	Pre-treated	Control	Pre-treated	Control	Pre-treated
20	90	110	230	1.61	1.72	1.01	.87	.73	.08
10	110	220	130	1.89	.89	1.11	.54	.52	.02
20	20	160	250	2.83	2.22	1.60	1.11	.86	.39
20	20	280	90	1.67	1.75	.98	1.16	.58	.93
20	30	110	10	1.61	.06	1.01	.05	.72	.04
20	180	150	10	1.39	.33	.79	.40	.44	.00
20	20	150	100	1.11	.25	.82	.18	.54	.14
20	20	180	30	1.61	.14	1.20	.14	.84	.07
20	30	110	120	1.22	.50	.73	.31	.52	.17
20	90	170	140	1.92	.86	1.42	.43	.88	.04
20		140		3.00		1.92		1.18	
30		160		1.64		1.19		.64	
20		190		1.97		1.23		.62	
Avg									
20	61	163.9	111.0	1.805	.872	1.155	.519	.698	.188
Probability									
<0.01		0.10		<0.01		<0.01		<0.01	
Significance									
High		None		High		High		High	

All temperatures are rectal and are expressed as increments above basal temperature determined during pre-pyrogen control period.

group of animals pre-treated with blocking agent. The average response of the pre-treated group is 73% less than that of the control. On closer inspection of the individual dogs in the group of animals pre-treated with blocking agent, it is apparent that only 2 of them showed febrile reactions of any consequence and the reaction can be said to have been essentially abolished in the remainder.

It is concluded that a potent adrenergic blocking drug reduces the febrile response to the injection of pyrogenic material. In most curarized dogs the reaction is nearly abolished and greatly delayed in onset, but in two of them a nearly normal pyrogenic reaction was observed.

Assuming that the present drug alters the pyrogenic reaction because of its ability to block adrenergic nerves, then partial inhibition of the febrile response to pyrogen by this drug may be interpreted in several ways. The adrenergic blocking action of the drug may be only partial, or adrenergic nerve mediated vasoconstriction may contribute only partially to the pyrogenic reaction.

The febrile response in curarized dog is due in large part to vasoconstriction. Thus, partial reduction of the pyrogenic reaction under these conditions implies either incomplete adrenergic blocking action on the part of the drug or participation in the febrile response of a non-sympathomimetic vasoconstrictor substance.

In line with the latter interpretation are the observations that complete sympathectomy modifies but does not prevent the pyrogenic reaction in the cat[14] and that after complete sympathectomy and adrenal inactivation, the ears of some rabbits still showed delayed vasoconstriction associated with the rise in body temperature.[8]

Summary. A potent adrenergic blocking drug, N-Ethyl-N-(2-bromoethyl)-1-naphthylenemethylamine · HBr, has been shown to reduce and alter the febrile response of normal and curarized dogs to the intravenous injection of a purified pyrogenic material obtained from pseudomonas aeruginosa. This evidence is interpreted as supporting the hypothesis that the pyrogenic reaction is due, at least in part, to a reduction in heat loss caused by adrenergic nerve mediated vasoconstriction in the skin.

16863

Comparison of RBC Counts in Central and Peripheral Blood in Various Laboratory Animals.*

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The observation that red cell counts of blood from the tail of the rat were higher than counts of blood from the larger vessels and heart was reported by Jolly.[1] Reichel and Monasterio[2] listed several authors who had found no difference between human arterial and venous red cell counts, nor between venous, ear, and finger samples. Haden and Neff[3] reported higher counts in peripheral than in longitudinal sinus blood of infants, but Lucas *et al.*[4] reported the opposite in the newborn.

Materials and methods. Two samples from each animal were taken with the same pipette, and each was counted in duplicate in a Neubauer-type hemacytometer. Hayem's solution was used as a diluting fluid. The significance of differences between central and peripheral counts was tested by Fisher's[5] "t" method of statistical treatment. All results are given in Table I.

Experiments and results. A total of 175 rats from 1 to 99 days of age was used. Tail vessels were dilated by warming at about 43°C for 30 seconds or less, and blood obtained by a transverse cut an inch or so from the distal end, following which a ligature was applied to stop bleeding. Central blood was procured by decapitation approximately one hour later. Averages of results indicate that peripheral blood had a higher red cell count than blood obtained by decapitation. However, statistical treatment indicates that this difference is not highly significant.

Thirty adult mice were sampled in the same way as rats. Warming the tail was omitted in 24 mice to determine whether this influenced red cell count, but no change was apparent. Tail and head samples did not differ significantly in red cell count in these animals.

Red cell counts in 13 Golden Syrian hamsters were made as described for mice. The difference between tail and head counts was found not statistically significant.

Twenty adult rabbits were anesthetized with 35 mg/kg sodium pentobarbital intravenously at least 30 minutes before sampling. Peripheral blood was obtained by nicking the ear margin, avoiding the larger vessels, and central blood secured about 20 minutes later by cardiac puncture. The order of sampling was reversed in half the animals. No significant difference between the red cell counts of the two samples was found.

The peripheral blood of 20 unanesthetized guinea pigs was obtained by nicking the ear after it had been warmed slightly under a lamp, and the central blood collected by cardiac puncture about an hour later. Red cell counts of ear and heart blood did not differ significantly.

Twenty adult dogs were anesthetized with 32.5 mg/kg sodium pentobarbital intraperitoneally at least 30 minutes before sampling. This interval permitted splenic engorgement, which influences blood concentration during this type of anesthesia, according to Carr and Essex.[6] Blood was taken from the tail and

* This work was aided by a grant from the University of Missouri Research Council.

† Present address: St. Louis University School of Medicine, St. Louis, Mo.

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TABLE I.
Summary of RBC Counts of Central and Peripheral Blood in Various Laboratory Animals.

Animal	Age	No. of counts ♂	No. of counts ♀	Avg RBC count of central blood, ×1000	Avg RBC count of peripheral blood, ×1000	Probability
Rat	1-99 Days	64	101	5,297	5,729	.1–.05
Mouse	Adult	15	15	9,648	10,571	.1–.05
Guinea pig	"	12	8	6,422	6,160	.4–.5
Hamster	"	5	8	8,283	9,388	.1–.05
Rabbit	"	11	9	6,265	6,396	.6–.5
Dog	1 day	3	7	4,864	5,166	.6–.5
	Adult	6	14	6,284	6,529	.5–.4

heart, with about 15 minutes elapsing between samples. The order of sampling was reversed in about half the dogs in order to minimize the effect of splenic engorgement.

Peripheral and central red cell counts were also made in 10 newborn dogs at 1 day of age. No significant difference between central and peripheral red cell counts was found in young or adult dogs.

Discussion. In the study of central and peripheral red cell counts in 6 species, only the rat, mouse and hamster showed a difference between the two samples that approached significance. The statistical method used indicated, however, that the differences found in the averages could have been due to chance, since the probability factor was between 0.1 and 0.05, whereas a factor of 0.05 to 0.01 is necessary to demonstrate a high significance.

Summary. Red cell counts of blood secured from peripheral vessels were compared to counts from the heart or large vessels in the rat, mouse, hamster, rabbit, guinea pig, and dog. In none of these was a statistically significant difference found.

[6] Carr, D. T., and Essex, H., *Am. J. Physiol.*, 1944, **142**, 40.

16864

Attempt at Percutaneous Introduction of d-Tubocurarine with a Direct Current into Muscles of Rabbits.*

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Curare affords a possible indicator to gauge the depth of penetration of positive ions through the skin under the action of a galvanic current (electrophoresis, iontophoresis), since 1) its physiological action is solely on muscle and can easily be detected, 2) it is active in small amounts, 3) it is dissociated, at least in acidic solutions, into positive ions suitable for transfer at the positive pole. Previous work on the depth of penetration using dyes (Harpuder,[1] Harpuder and Rein[2]) have shown visible dye only in the epidermis in humans, and in dogs and rabbits only a faint coloration of the superficial muscle layers under extreme conditions. The possibility remains, however, that in deeper layers the dye was decolorized or otherwise made undetectable as rapidly as it entered them.

* This research was aided by a grant to Columbia University by the Baruch Committee for Physical Medicine.

† Columbia Baruch Fellow in Physical Medicine.

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[2] Rein, H., *Z. f. Biol.*, 1926, **84**, 41.

TABLE I.

Threshold Values* of Single and Tetanizing Shocks for Peroneal-Dorsi-Flexor System Before and Following Electrophoresis with *d*-Tubocurarine.

Control leg				Test leg				pH of solution	Remarks
Single stimulus		Tetanic stimuli		Single stimulus		Tetanic stimuli			
a	*p*	*a*	*p*	*a*	*p*	*a*	*p*		
15	15	11	12	12½	12½	10	8½	4	Control: wet dressings
12½	12½	8½	8	15	15	12	12½	4	with tubocurarine
20	17½	15	15	12½	12½	12½	12½	4	Control: tubocurarine-
15	12½	10	10	8	7	6	7	4	free solution and elec-
20	20	17½	16	17½	17½	15	15	7	trophoresis
17½	20	15	16	22½	20	17½	15	7	
Avg $(p-a)$		Avg $(p-a)$		Avg $(p-a)$		Avg $(p-a)$			
+0.4		0		−0.6		−0.4			

* Threshold units are arbitrary, determined by ohmic resistance, and are proportional to intensity of stimulus.

a = before electrophoresis.

p = following electrophoresis.

Therefore, the present work using curare as a physiological rather than a histological indicator in the muscles is in order.

Nerve muscle preparations *in situ* were employed in rabbits anesthetized with sodium pentobarbital (30 mg/kg). The sciatic nerves were exposed bilaterally, the tibial nerves sectioned, and electrodes placed on each peroneal nerve. Upon electrical stimulation of the nerve, activity of the anterior compartment of muscles of the lower leg was set up to be recorded on a kymograph as dorsiflexion of the ankle joint. The upper legs were rigidly fixed by means of pins passed through the lower ends of the femora. Interference with circulation was avoided.

Electric contact of the positive electrode over the lower legs (hair clipped off) was by means of gauze pads soaked in a test solution and covered with a metal strip. The negative electrode was a large saline-soaked gauze pad placed over the abdomen (likewise with hair clipped off). The test solution containing d-tubocurarine‡ was made up to a concentration of 143 mg % in 50% ethyl alcohol containing 1:20,000 adrenalin. Alcohol was used because of the evidence that alcoholic solutions favor electrosmosis of positive ions through the skin,[3] and adrenalin because it was hoped that by sub-papillary vasoconstriction it would minimize removal of d-tubocurarine by the blood stream.[4] In 4 experiments the pH of this solution was approximately 4, and in 2 it was adjusted to approximately 7, to favor electroendosmosis through the epidermis.

The experimental plan was to utilize one leg as a test leg, the other as a control. Two types of control were employed. In the first type (4 observations) 15 ma. of direct current was passed through each leg for 20 minutes, but the solution over the control leg contained no d-tubocurarine, only alcohol, adrenalin and one drop of 0.85% NaCl. In the second type (2 observations) solutions containing d-tubocurarine were placed over both legs, but current was passed through only one. Thus, in the first type of control the only variable between the two legs was the presence of d-tubocurarine; in the second type the variable was the passage of current.

Using an electronic stimulator[5] which afforded graded intensity of stimuli of constant duration, the thresholds to a single shock and tetanizing stimuli (50 per second) were determined by recording the response on the kymograph immediately before the passage of the direct current through the legs and

‡ We are indebted to the Abbott Laboratories, North Chicago, Ill., for a supply of this material.

[3] Rothman, S., *J. Lab. and Clin. Med.*, 1943, **28**, 1305.

[4] von Sallmann, L., *Arch. Ophthl.*, 1943, **29**, 711.

[5] Nastuk, W. L., and Borison, H. L., *Rev. of Scient. Instruments*, 1947, **18**, 669.

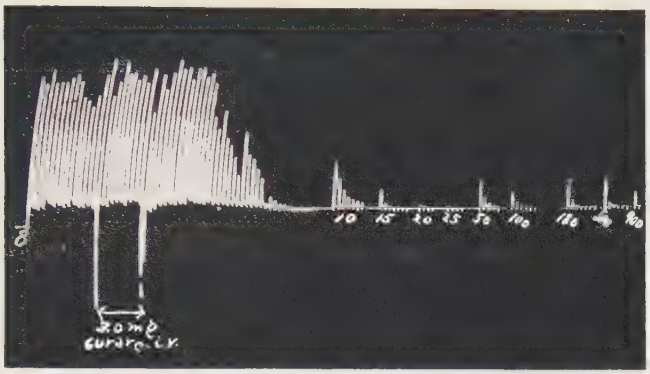

FIG. 1.
Effect of an intravenous curarizing dose upon threshold value of single electrical shocks in a nerve-muscle preparation.
Time scale = one stimulus per second. Regular stimulus at threshold (8 units) was begun, curare was injected and strength of stimulus increased as response fell (all indicated in subscript figure on tracing).

again immediately after the completion of flow of this current for a period of 20 minutes.

Threshold values to a single electric shock or tetanic stimuli remained unaltered in the control leg both after the simple application of d-tubocurarine wet dressings and electrophoresis with a d-tubocurarine-free solution. Similarly, the test leg showed no demonstrable change in thresholds following electrophoresis with the d-tubocurarine solution (Table I). Thus, within the limits of our method, such treatment does not introduce detectable amounts of the drug. It remains to measure the order of magnitude of tubocurarine in muscle that can be detected by our method.

A separate experiment using intravenous d-tubocurarine indicated that the presence of minute amounts of tubocurarine could be readily detected in the muscle. Fig. 1 illustrates the extreme degree of loss of excitability to electrical stimulation of our nerve-muscle system in a 3 kg rabbit receiving 3.0 mg of tubocurarine intravenously. Assuming a total muscle weight of 1.35 kg and 5 g as the weight of the muscles actually tested, then a maximum of 0.011 mg of tubocurarine reached the muscle. This value is an overestimation, since some of the drug injected must remain in the body fluids outside the muscles. Thus, our method is sensitive to less than 0.011 mg of tubocurarine.

Simple calculation (on the basis of electrochemical equivalents) shows that the passage of 15 ma for 20 minutes represents the ionic transfer of 0.1865 m.eq. of a monovalent positive ion or 58.2 mg in terms of tubocurarine.§ Since less than 0.011 mg (0.000035 m.eq.) of the drug can be detected by our method, to curarize our preparation by electrophoresis less than 1/5300 of the current need be transported by the tubocurarine ion, once it has crossed the skin barrier. Our results indicate that even such small amounts could not be introduced by the electric current. Thus, it appears that penetration of tubocurarine, to the muscle layer, if it occurs at all, yields concentrations too minute to be physiologically active.

Summary and conclusions. The question of the effective depth of percutaneous penetration of substances introduced by direct galvanic current was explored, utilizing the effect of d-tubocurarine upon muscle as an indicator system.

Observations on 6 rabbits indicated that threshold values to electrical stimulation of a suitable nerve muscle preparation was not altered after electrophoresis with d-tubocurarine.

Curarization of a rabbit with an intraven-

§ 1 m.eq. tubocurarine = 312 mg.

ous dose revealed that to achieve an effect with tubocurarine electrophoresis only a maximum of 0.011 mg tubocurarine need reach the muscle.

Thus, further evidence is adduced that penetration of substances through the skin by electrophoresis is limited.

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In vitro Effect of Certain Antibacterial Agents on Organisms Encountered in Bovine Mastitis.*

MARY E. KRAFT AND G. R. SPENCER. (Introduced by S. H. McNutt.)

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In screening materials to learn which gave promise in the treatment of bovine mastitis, the *in vitro* effect of a number of antibiotic substances and sulfonamides was determined on some of the bacteria frequently associated with cases of bovine mastitis.

Some information is already available in the literature relative to the materials employed. Salle and Jann[1] reported that subtilin was bacteriostatic in high dilutions and bactericidal in lower dilutions for gram positive organisms, including *Mycobacterium tuberculosis*. Buggs and co-workers,[2] working with streptomycin *in vitro* found a great range in susceptibility of different strains of the same organism.

Goetchius and Lawrence[3] found that 3′-5′ dibromo sulfanilanilide *in vitro* was effective in high dilution against *Streptococcus agalactiae, Streptococcus viridans,* and *Brucella abortus*. Schweinberg and Yetwin[4] stated that sulfamethazine *in vitro* was more bactericidal and bacteriostatic than sulfadiazine or sulfamerazine against *Eberthella typhosa, Escherichia coli,* and certain Salmonella. Francis *et al.*[5] reported that 4, 4′ diaminodiphenyl sulfone (hereafter designated sulfone) showed promise against *Str. agalactiae* infections in the chick embryo and mouse. Burbaum and others[6] found that sulfadiazine was slightly inhibitory against *Corynebacterium diphtheriae.*

Methods. The organisms used in these experiments were *Str. agalactiae, Br. abortus, Pseudomonas aeruginosa, E. coli,* a coagulase-positive *Staphylococcus aureus,* and *Corynebacterium pyogenes. Salmonella typhimurium* was employed as a representative of the Salmonella.

A culture of each of the above organisms was first grown in tryptose broth for 24 hours at 37°C. Inocula for the actual tests were prepared from such broth cultures. The *C. pyogenes* broth culture was used undiluted while the others were diluted 1 to 100 in broth. Various concentrations of each of the antibacterial agents were prepared in 10 ml of sterile skimmed milk to which brom cresol purple was added as an indicator, and these tubes were inoculated with 0.2 ml of the suspensions of the organisms. Those cultures producing no visible change in milk were streaked on blood agar or nutrient agar slants to determine the extent to which the culture had grown in the presence of the agent. Thus the tests were carried out in skimmed milk

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station as a contribution from project number 475.

1 Salle, A. J., and Jann, G. J., *J. Bact.*, 1946, **51**, 592.

2 Buggs, C. W., Bronstein, B., Hirshfeld, J. W., and Pilling, M. A., *J.A.M.A.*, 1946, **130**, 64.

3 Goetchius, G. R., and Lawrence, C. A., *J. Lab. and Clin. Med.*, 1946, **31**, 336.

4 Schweinberg, F. B., and Yetwin, I. L., *J. Bact.*, 1946, **49**, 193.

5 Francis, J., Peters, J. M., and Davies, O. L., *J. Comp. Path.*, 1947, **57**, 162.

6 Burbaum, L., Nenner, N., and Dolgopol, V. B., *J. Bact.*, 1947, **53**, 507.

TABLE I.
Dilutions of Subtilin Necessary for Bactericidal and Bacteriostatic Action After 72 Hours Incubation.

Effect	*S. aureus*	*Str. agalactiae*	*C. pyogenes*
Bactericidal	1:80,000	1:80,000	1:100,000
Bacteriostatic	1:160,000	1:160,000	1:200,000

Subtilin lot 205-0, potency equivalent to 200, was obtained through the kindness of Dr. Hans Lineweaver, Western Regional Laboratory, Albany, Calif.

TABLE II.
Effect of Streptomycin on the Various Organisms After 72 hours Incubation, Reported as Units per ml.

Effect	*S. aureus*	*Str. agalactiae*	*C. pyogenes*	*S. typhimurium*	*E. coli*	*Br. abortus*	*Ps. aeruginosa*
Bactericidal	50	50	25	500	62.5	25	1000
Bacteriostatic	25	25	12.5	250	—	12.5	—

Streptomycin, lot No. 544, containing 573 units/mg, was obtained through the courtesy of Merck & Co., Rahway, N.J.

TABLE III.
Effect of Sulfonamides on the Organisms After 24 Hours Incubation.

Sulfonamide	Mg % conc.	*S. aureus*	*Str. agalactiae*	*C. pyogenes*	*S. typhimurium*	*E. coli*	*Br. abortus*	*Ps. aeruginosa*
Control		3+	3+	3+	3+	3+	3+	3+
3′-5′-dibromo-sulfanilanilide	8	2+	3+	3+	3+	3+	3+	3+
Sulfamethazine	8	3+	+	+	3+	—	—	3+
	1.6	3+	2+	+	3+	+	2+	3+
Sulfapyridine	8	2+	—	—	2+	±	+	±
	1.6	3+	+	3+	3+	+	+	2+
Sulfone	8	2+	±	—	3+	2+	+	3+
	1.6	3+	+	3+	3+	3+	2+	3+
Sulfadiazine	8	2+	±	—	2+	±	—	—
	1.6	3+	+	3+	3+	±	+	—
Succinyl sulfanilamide	8	3+	3+	3+	3+	3+	3+	3+
Sulfamerazine	8	2+	—	—	3+	—	—	±
	1.6	3+	±	—	3+	±	+	2+

— Complete inhibition of growth in milk, organisms not killed.
± Slight growth of organisms in milk.
+, 3+ Relative amounts of growth.

to provide somewhat the same medium in which the organisms are found growing in the bovine udder. The organisms were diluted to the approximate number likely to be found in mastitis.

Likewise, the various antibacterial agents were diluted to the approximate level which could be attained for a period of 10 to 12 hours after intramammary infusion in cows. The agents tested were subtilin, streptomycin, 3′-5′ dibromo sulfanilanilide, sulfamethazine, sulfapyridine, sulfone, sulfadiazine, succinyl sulfanilamide, sulfamerazine, disodium sulfone, and sodium sulfamethazine. The lowest concentration which prevented visible growth was termed the bacteriostatic concentration: that which killed all organisms, the bactericidal concentration.

Results. In Table I the results of studies on subtilin are compiled. *S. aureus* and *Str. agalactiae* were slightly more resistant than *C. pyogenes*. Table II shows the bacteriostatic and bactericidal concentration of streptomycin for the seven organisms. *Ps. aeruginosa* and *S. typhimurium* were much more resistant than the other organisms. In Table III the effects of various sulfonamides on the different organisms are shown. The 3′-5′ dibromo sulfanilanilide and succinyl sulfanilamide were not inhibitory in the amounts used; their bacteriostatic concentration is more than 8 mg %. Sulfamerazine and sulfadiazine

TABLE IV.
Effect of Disodium Sulfone on Various Organisms After 72 Hours Incubation.

Mg % conc. of sulfone	*S. aureus*	*Str. agalactiae*	*C. pyogenes*	*S. typhimurium*	*E. coli*	*Br. abortus*	*Ps. aeruginosa*
2270	—	—	—	±	±	—	±
1135	0	0	0	0	0	0	3+
227	±	±	±	2+	±	+	0
114	2+	0	+	3+	+	2+	0
23	0	±	0	0	0	0	0
11	0	+	0	0	0	0	0
Control	3+	3+	3+	3+	3+	3+	3+

0—No determination run.
— No growth, culture killed.
± Slight growth with no visible change in milk.
+, 3+ Relative amount of growth.
The 2.27% solution of disodium sulfone was obtained through the courtesy of Dr. C. A. Woodhouse from Dupont of Delaware, Md.

TABLE V.
Effect of Sodium Sulfamethazine on *S. agalactiae* and *E. coli*.

Mg % conc. of drug	Hours incubation	*St. agalactiae* strains				*E. coli*
		1723	MP5	24D	N-6	MP 8
2500	24	—	—	—	—	—
	48	+	+	+	+	+
250	24	3+	+	3+	2+	+
	48	3+	3+	3+	3+	3+
25	24	3+	3+	3+	2+	+
	48	3+	3+	3+	3+	3+
2.5	24	3+	3+	3+	2+	+
	48	3+	3+	3+	3+	3+
Control	24	3+	3+	3+	3+	3+

1723, MP5, 24D, and N-6; *S. agalactiae* cultures from cows with chronic mastitis.
MP8—*E. coli* culture from a cow with acute mastitis.
— No change in milk.
+ Slight acid in milk.
2+ Acid in milk.
3+ Acid and coagulation in milk.

were active against *E. coli, Br. abortus, Str. agalactiae,* and *Ps. aeruginosa.* They appeared to have a wider range of action than the other sulfonamides. Sulfapyridine and sulfamethazine were slightly less active. Sulfone was much less active against the gram negative organisms. The strains of *S. aureus* and *S. typhimurium* were resistant to the sulfonamides. In Table IV the effect of disodium sulfone is presented. The concentration required for inhibition of growth was more than 227 mg % for all 7 strains. Disodium sulfone was more active against *Str. agalactiae* than against the other organisms. As shown in Table V sulfamethazine sodium inhibited the 4 *Str. agalactiae* cultures and one *E. coli* culture for 24 hours in a 2500 mg % concentration but permitted growth in this concentration in 48 hours. *E. coli* was slightly more sensitive than strains of *Str. agalactiae.*

Discussion and summary. Data are presented on the effect of various antibacterial agents on a representative group of organisms associated with cases of chronic and acute mastitis.

Subtilin was effective in high dilution against the gram positive organisms. Streptomycin appeared to more active against the gram positive cultures than against the gram negative organisms. Strain variations among cultures of the same species of organism may

have been responsible for the poor action of streptomycin against these particular gram negative organisms.

The strains of *S. aureus* and *S. typhimurium* were resistant to the sulfonamides employed. *Str. agalactiae* and *C. pyogenes* were susceptible to sulfapyridine, sulfone, sulfadiazine and sulfamerazine. *In vivo* study is necessary to prove these drugs of value as adjuncts to penicillin in the treatment of chronic streptococcic mastitis and so-called "summer mastitis." *E. coli* was sensitive to sulfapyridine, sulfamethazine, sulfadiazine, and sulfamerazine among which sulfamerazine and sulfamethazine showed great activity. *Br. abortus* was susceptible to sulfamethazine, sulfadiazine, and sulfamerazine. *Ps. aeruginosa* was inhibited by sulfamerazine and sulfapyridine. Against the strain of *Ps. aeruginosa* employed, sulfadiazine was the most effective antibacterial agent.

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The Effect of Heparin on Cell Division.*

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When a living cell is incited to divide, its protoplasm first undergoes a sharp increase in viscosity. There is indeed what may be called a mitotic gelation and this gelation precedes the appearance of the mitotic spindle. Once the spindle is formed, the protoplasm reverts to its original fluid state. This sequence of stages in the protoplasm of a dividing cell has been described by all authors who have used objective methods for the determination of protoplasmic viscosity, (for discussion, see Heilbrunn;[1] Heilbrunn and Wilson[2]).

Since 1928,[1] it has been maintained that the mitotic gelation is similar to the gelation produced in protoplasm by so-called stimulating agents, and that this fundamental gelation reaction of protoplasm is in many respects similar to the gelation that occurs in vertebrate blood when it clots. As a matter of fact the protoplasm of all types of living cells is rich in thrombin or thromboplastic substances, substances which affect not only protoplasm but blood as well (compare Heilbrunn *et al.*[3] and references cited there).

If this line of reasoning is correct, it might be thought that substances which inhibit blood clotting would also prevent protoplasmic clotting in general and mitotic gelation in particular. Clearly, then, a substance like heparin is of interest in this connection.

Perhaps the best type of cell in which to observe protoplasmic clotting is the egg of the sea-urchin Arbacia. When this cell is torn or broken, as the protoplasm begins to flow out, the cell seals itself in a reaction like that which occurs when blood flows from a vessel.[4] This reaction has been called the surface precipitation reaction. Years ago, one of us attempted to show that heparin might have some effect on this surface precipitation reaction. However, no effect could be observed. Because of this failure to demonstrate an effect of heparin on the surface precipitation reaction, no further studies were made. Moreover, it was hardly to be expected

* Aided by a grant from the U. S. Public Health Service.

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that heparin could act on the interior of a cell, for the heparin molecule is a large one and it scarcely seemed plausible to suppose that heparin could enter a cell in sufficient amount to affect clotting processes in the interior protoplasm.

Fortunately for us, some months ago, one of our beginning graduate students, Mr. Julius H. Jacobson, was less certain of failure and in some preliminary experiments was able to show that heparin tended to prevent clotting in the protoplasm of paramecium. Following this lead we took courage and began to wonder whether heparin might not have an effect on the mitotic gelation as it occurs in marine eggs.

In a recent paper[2] we have shown the exact course of the mitotic gelation in the egg of the worm Chaetopterus and the time sequence of this gelation in relation to the morphological changes which take place during cell division. Hence it was logical to attempt a study of the effect of heparin on various aspects of mitosis in the Chaetopterus egg.

Unfortunately, no very pure preparations of heparin were available to us. We began work with two different lots of the Hynson, Westcott and Dunning preparation. In preliminary experiments with sea-urchin (Arbacia) eggs, we found that one of these lots (no. 194) was apparently contaminated with thromboplastin or thromboplastic substance. When sea-urchin eggs are placed in oxalate solutions and then broken by pressing down on the coverslip over them, no surface precipitation reaction occurs and instead of the protoplasm sealing itself as it does when calcium is present, it flows out in a steady stream. On the other hand, if to the oxalate solution an extract of injured living material is added, the protoplasm even in the absence of calcium does show a surface precipitation reaction.[3] Thus the protoplasm of the sea-urchin egg can be used as an indicator for the presence of clotting (thromboplastic) substances. A drop of Arbacia eggs in sea-water was placed on a slide; to this was added a drop of 0.45 molar potassium oxalate and a drop of 0.25% solution of heparin from lot 194 of Hynson, Westcott and Dunning. After waiting a minute for the calcium of the sea-water to precipitate out, the eggs were crushed. A definite surface precipitation reaction occurred. The experiment was repeated many times, always with the same result. The result indicates the presence of a protoplasmic clotting substance in the heparin. An alcoholic extract of the heparin was prepared. This was evaporated to dryness and the dry residue dissolved in distilled water. The protoplasmic clotting substance was present in the alcoholic extract. Years ago, in preparing heparin from lung tissue, Charles, Fisher and Scott[5] found that it was difficult to separate the heparin from a substance in lung tissue which favored blood clotting; this clotting substance was soluble in alcohol. The presence of a clotting substance in lot 194 of the Hynson, Westcott and Dunning heparin was also indicated by the fact that dilute solutions (0.1 - 0.02%) also caused a well-marked increase of the protoplasmic viscosity of the protoplasm of sea-urchin eggs exposed to these solutions for 2 hours. Viscosity tests were made with the centrifuge method.

Fortunately, another lot of the Hynson, Westcott and Dunning heparin was free from the clotting substance. This was lot 198. Solutions of it did not induce a surface precipitation reaction in the presence of oxalate nor did they cause increase in protoplasmic viscosity. Accordingly, in our experiments on the effect of Hynson, Westcott and Dunning heparin on Chaetopterus eggs, we were always careful to use lot 198.

When Chaetopterus eggs are placed in dilute heparin solutions and are inseminated some minutes later in the usual way with dilute suspensions of spermatozoa, they typically do not cleave. Much of this effect is due to an inhibition of fertilization by the heparin. Unfortunately, fertilization in the Chaetopterus egg is not accompanied by any marked cortical change, so that it is not possible to tell immediately whether or not sperm entrance has been effected. However, at about 9 minutes after fertilization (at 21°) the first polar body is given off, so that a count of

[5] Charles, A. F., Fisher, A. M., and Scott, D. A., *Proc. and Trans. Roy. Soc. of Canada*, 1934, 3rd ser., **28**, sec. V, 49.

polar bodies can indicate whether or not sperm entrance has occurred. Because the polar bodies of Chaetopterus are rather small, it is not easy to make polar body counts. We made counts in two ways. If the eggs are undisturbed during the count, ordinarily only about a third of the polar bodies are visible, so that a count which showed approximately 35% of the eggs with polar bodies would indicate that all of the eggs had been fertilized. If the eggs are rolled during counting, as high as 90% of the eggs could be seen to have polar bodies. Ordinarily, we did not roll the eggs, and in calculating percentages of eggs with polar bodies, we assumed that a 35% count indicated that 100% of the eggs had polar bodies.

By making counts of polar bodies we were able to determine with some certainty whether or not sperm entrance had occurred in any given case. After some disappointing experiments, we were fortunate to find that by increasing the concentration of sperm we could override the inhibiting effect of heparin on fertilization. In some cases in which sperm entrance did not occur (as judged by the polar body test) we were able to induce sperm entrance by adding more sperm. As an example of the effect of sperm concentration, the following experiment may be cited. A drop of dry sperm, obtained by cutting a worm, was diluted with 10 ml of sea-water to form a sperm suspension. Varying amounts of this sperm suspension (1, 10, 20 drops) were added to eggs which had been for 10 minutes in a 0.05% solution of heparin in sea-water. The results are shown in Table I. In this table percentages of eggs with polar bodies are calculated as noted above. It is clear that higher concentrations of sperm cause an increased percentage of sperm penetration.

If sperm penetration has occurred and then the eggs do not cleave, it is obvious that heparin has prevented the division of the cell. Table II shows a series of 10 experiments. In all of these experiments, control eggs inseminated in sea-water showed approximately 100% fertilization and cleavage. The experimental eggs were inseminated after 10 minutes immersion in 0.05% heparin solutions. Polar body counts showed the approximate percentage of eggs in which sperm penetration had occurred, calculated as before. Of these fertilized eggs, only a relatively small percentage showed cleavage. From the table it is obvious that dilute solutions of heparin can block mitosis in the Chaetopterus egg.

TABLE I.
Effect of Increasing Concentrations of Sperm on Sperm Entrance Into Heparinized Eggs as Indicated by Polar Body Formation.

No. of drops of sperm suspension added	% of eggs with polar bodies
1	3
10	51
20	57

TABLE II.
Effect of Heparin on Cleavage.

% of eggs with polar bodies	% of eggs cleaved	% of fertilized eggs cleaved
100	27	27
86	8	10
74	22	37
51	25	49
57	10	18
51	36	71
63	6	10
100	13	13
91	42	46
100	61	61

In Table II the first 8 experiments were done with Hynson, Westcott and Dunning heparin (lot 198). In the final 2 experiments, Hoffmann-LaRoche heparin was used. Other experiments in which eggs were exposed to heparin for longer times indicated that Hoffmann-LaRoche heparin is weaker in its effects than Hynson, Westcott and Dunning heparin. Fortunately, however, the Hoffmann-LaRoche heparin does not appear to contain any thromboplastic substance.

The effect that heparin has on cell division is not due to a killing action; at any rate the effect is, to some extent at least, reversible. Thus in one experiment in which eggs treated with heparin for 10 minutes before fertilization showed 36% cleavage, washing the eggs several times in sea-water caused the percentage of cleavage to increase to 55%.

Apparently, heparin acts by preventing the mitotic gelation which normally occurs in the early stages of mitosis. In the Chaetopterus

egg, during the mitosis which causes the division of the single egg cell into two blastomeres, the protoplasmic viscosity rises sharply just before the mitotic spindle is formed. This is clearly shown in the graph published recently.[2] Before the mitotic gelation, the protoplasmic viscosity has an arbitrary value of 7; this rises to 14 and then after the spindle is formed it drops to 7 again. In the heparinized eggs, such a mitotic gelation does not occur. Five tests of the protoplasmic viscosity of fertilized heparinized eggs showed in every case a viscosity of 6 or below. These tests were all made from 70-90 minutes after fertilization, that is to say, after ample time had been allowed for the mitotic gelation to occur. Thus beyond much doubt heparin prevents mitosis by inhibiting the mitotic gelation.

The fact that heparin can prevent cell division is not a new observation. Years ago Fischer[6] showed that heparin could prevent the division of cells in tissue culture. Presumably in these cells also heparin acts by preventing protoplasmic clotting. The concentration of heparin that Fischer used in his later experiments is identical with that we found best for Chaetopterus eggs. However, Fischer apparently used a purer preparation of heparin than the one we had at our disposal.

An important fact to remember is that heparin is a normal constituent of the blood and perhaps also of the cells of higher animals. It has been stated that in radiation sickness there is an increased amount of heparin in the blood.[7] Conceivably some of the effect of roentgen rays on cancer might be due to the presence of this heparin and its effect on cell division. Also it should be noted that the bacterial polysaccharide which in minute amounts causes regression of tumors in rats and mice is a hemorrhagic agent.[8] Inasmuch as excessive doses of heparin are known to induce hemorrhage,[9] the bacterial polysaccharide may perhaps be related to heparin. It may also be possible that the injection of one type of heparin or another, or the injection of other types of substances which impede blood clotting, like injection of dicumarin, may eventually prove to be of some help in the prevention of excessive cell division such as occurs in cancer.

Recent experiments in our laboratory by Drusilla Harding have shown that heparin can prevent cell division in the frog egg. These experiments will be published before long. We are also experimenting on the effect of injecting heparin and other anti-coagulants on sarcoma in mice.

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Heparin and Heparinocytes in Elephantiasis Scroti.

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Through the brilliant researches of Jorpes[1] and Holmgren and Wilander[2] it has been well established that the mast cells or heparinocytes are cellular sources of heparin. However, heparin of the basophilic granules is not identical with circulating heparin or heparin extracted from tissues. The mast cell heparin is monosulfuric acid, and when released is transformed into di, tri, and tetrasulfuric acids, circulating or tissue heparin being a mixture of all of these elements.[3,4]

The distribution of heparinocytes in the tissues varies considerably in different species. In dogs, abundant heparinocytes are found in the lobules and veins of the liver, whereas in rabbits and rats they are scanty or absent here. In rats, abundant heparin is found in the skin. The intestines of the various animals all contain many heparinocytes; the lungs show a moderate number, and the spleen only a few. Rats contain fewer but larger heparinocytes than rabbits and dogs.[2,5]

It has been shown that heparin and similar compounds if given intravenously are taken up by the macrophages, particularly of the liver, spleen and lymph nodes, and that some is excreted through the kidneys.[6] That this is not the cause for the dissipation of heparin is apparent from studies involving splenectomy; complete hepatectomy; combined nephrectomy, splenectomy, and hepatectomy; injections into the bone marrow of the tibia; ligation of the carotid and vertebral arteries; blockade of the reticulo-endothelial system with India ink; stimulation of the reticulo-endothelial system by histamine, and depression of it by antihistaminic drugs.[7,8]

The pathology of the heparinocytes has hardly been studied. It is known only that the tissue mast cells are greatly increased in urticaria pigmentosa in man[9] and in solitary "mastocytomas" in man[9] and in dogs.[10] Oliver, Bloom and Mangieri,[11] who recently analyzed two dog mastocytomas chemically, found 20.1 g of a crude heparin in 330 g of tissue from a well differentiated tumor; from this they extracted 2,460,000 A.C.U. or 492,000 I.U. of heparin per kilogram of tumor, *i.e.*, 50 times as much as found in the liver of normal dogs. Paff, Bloom and Reilly[12] succeeded in cultivating these mast cells; they made the interesting observation that only mast cells grew in the cultures, as though the product elaborated by these cells prevented growth of other cells.

The present report deals with mast cells and heparin in a human scrotum amputated because of elephantiasis. The study was prompted by the observation of absence of thrombosis in this disease. It was speculated that coagulation of lymph and blood in the obstructed tissue was possibly prevented by excess heparin produced locally.

The owner of the scrotum, a white pastry

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FIG. 1.
Abundant mast cells (heparinocytes) between distended lymph spaces.

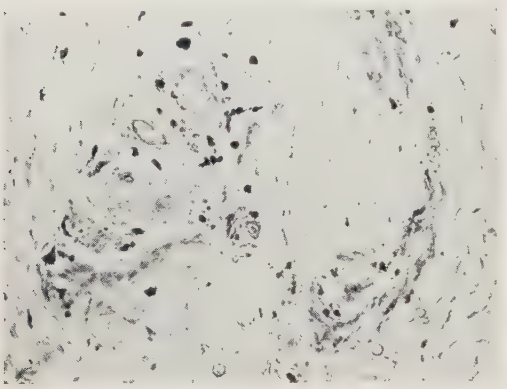

FIG. 2.
Abundant mast cells (heparinocytes) around blood and lymph vessels.

chef 55 years of age, came from Italy when he was 10. At 24, draft board examiners noted firm masses in both inguinal regions. When he was 43, the patient observed enlargement of the scrotum. He visited a physician only when the scrotum weighed over 50 lbs. and almost touched the floor. There was an invagination where the penis would normally be. The skin of the legs was congested and slightly cyanotic. There were superficial varicosities of all leg and thigh veins. The bleeding time was one minute, and the clotting time 5½ minutes.

Microscopic examination of tissue from the scrotum revealed numerous heparinocytes in the neighborhood of lymph and blood vessels (Fig. 1 and 2).

From 10 kg of tumor tissue, 11.11 g of crude heparin were extracted, following described methods.[13,14] Preparation of both the barium and the sodium salt yielded four fractions totalling 1.4 g of purified material. Plasma clotting studies were performed on thawed citrate dog plasma, using the photoelectric method as described by Foster.[15] One fraction of the heparin preparation in a concentration of 30 gamma per ml inhibited the clotting of plasma for 10 minutes, as opposed to 2½ minutes for the normal. Thirty gamma of a second fraction per ml prolonged the clotting of rabbit whole blood for 385 minutes, as compared with a control clotting time of 4 minutes. Intravenous injection of 5 mg of tumor heparin per kg into rabbits produced marked anticoagulation which reached its maximum within 15 minutes. A blood sample taken 15 minutes after the injection clotted in 65 minutes. A sample taken an hour after injection clotted in the normal time of 3 minutes.

The most potent fraction both *in vitro* and by direct intravenous injection into rabbits was of the same potency as the best commercial heparin currently used in clinical practice. The remaining fractions showed activity to a lesser degree. The fact that these fractions were not capable of purification to higher potency probably resides in the fact that most of the material was obtained from the mast cells and corresponded to Jorpes' lower sulfur-containing sulfuric acids, as the mono- and the di-sulfuric acid.

Summary and conclusions. Microscopic examination of a human scrotum amputated because of elephantiasis showed numerous heparinocytes around lymph and blood vessels. One hundred and twenty-six mg of purified heparin per kg of wet tissue was obtained. Based on the purified material, the scrotum contained at least 16,380 I.U. of activity per kg of fresh tissue. These findings are further evidence that mast cells are sources of heparin. They also explain the absence of thrombosis in elephantiasis.

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Survival Differences Breathing Air and Oxygen at Equivalent Altitudes.*†

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It can be shown on theoretical grounds[1] that when equivalent altitudes are chosen where the pO_2 of the inspired gases are identical breathing air and breathing 100% oxygen the resultant *alveolar* pO_2 in the lung are never the same unless the respiratory quotient is equal to unity. For example, if the alveolar pCO_2 is 40 mm Hg and the R.Q. = .8, the alveolar pO_2 is 8 mm lower breathing air than breathing 100% oxygen. Under the same conditions but with the R.Q. = 1.0 the alveolar pO_2 is the same. When the R.Q. is greater than 1.0 the alv. pO_2 is larger breathing air than breathing 100% oxygen. Thus at equivalent inspired O_2 tension altitudes the relative advantage or disadvantage of breathing air as compared to 100% oxygen is a function of the R.Q. This phenomenon has been referred to as the *R.Q. Effect* or *Nitrogen Dilution Effect* (the latter applies more specifically to conditions of R.Q. greater than unity). Acute exposure to low oxygen concentration induces hyperventilation which raises temporarily the respiratory quotient above 1.0. As long as the quotient remains above 1.0 it would be preferable to breath air rather than 100% oxygen at equivalent inspired O_2 pressures. Theoretically an advantage of only a few mm in alveolar pO_2 pressure is expected, but since in very low hypoxic environments a few mm pO_2 spell the difference between consciousness and unconsciousness it was felt that this nitrogen dilution effect might be demonstrable.

Method. Mice were conditioned to sit on a horizontal bar of ½" diameter suspended about 8 cm above the base of a large desiccator. The bottom was provided with a metal grid which electrically shocked the animal whenever it left the bar. Mice are very readily conditioned to this procedure and will immediately jump on the bar even when no current is sent through the grid. *Unconsciousness* was designated as that state where mice were no longer able to maintain themselves on the bar. This criterion has proven to be a very satisfactory index. The oxygen pressure was reduced by evacuating the desiccator by a pump. The rates of ascent on air and 100% O_2 had to be very accurately controlled so that the rates of change of the inspired pO_2 (B.T.P.S.) were identical in the two runs. Thus the timing of air runs started at ground level where the inspired pO_2 was .209 x (B-47). In the 100% O_2 runs the desiccator was first thoroughly flushed with oxygen for several minutes. This was followed by a slow ascent to an altitude of approximately 32900 ft. to allow for denitrogenation. The timing of the pure oxygen runs was started at this altitude where the inspired pO_2 (equal to B-47) was the same as at ground level.

Usually 6 mice were "taken up" at one time and the inspired pO_2 was calculated from the altitude at which unconsciousness set in. The air temperature on all runs was kept between 22.9 and 24.1°C.

Since the N_2 dilution or R.Q. effect is a transient affair depending upon the degree of hyperventilation and the magnitude of the CO_2 reserve of the blood and tissues, the rate of ascent was varied as indicated in Table I. The rates of ascent so listed are based upon 100% oxygen runs which started at 32900 ft. The ascents on air were adjusted in such a

* This work was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of Rochester. For continued support since 1946, we are indebted to a contract with the Air Materiel Command, Wright Field, Dayton, Ohio.

† The authors are greatly indebted to Mr. James Holmes for his technical assistance.

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TABLE I.
Oxygen Tension (B.T.P.S. —37°C) of the Inspired Gas at Which Mice Developed Unconsciousness Breathing Air and Pure Oxygen.

Series	Rate of ascent 10^3 ft./min.	pO_2 on 100% O_2 mmHg	S.D. mmHg	n	pO_2 on air mmHg	S.D. mmHg	n	Δ mmHg	S.E.D. mmHg
I	0.5	23.3	12.2	23	24.6	1.7	23	—1.3	2.40
II	2.	41.7	7.6	33	32.5	5.6	35	9.2	1.63
III	4.	39.8	7.0	28	31.0	3.5	33	8.8	1.45
IV	16.	40.8	6.5	22	31.7	3.7	22	9.1	1.60

fashion that the inspired pO_2 at any time was equal to that of the O_2 runs.

Results and Discussion. Four different rates of ascent were tried. In Series I it took on the average 42 minutes to reach the unconsciousness level and there was no significant difference between the two runs. It might be argued that during this long interval the N_2 dilution effect was nearly over (the CO_2 reserves used up) as has been found for altitudes of 18-22000 in man.[2] Had a still slower rate of ascent been used where the R.Q. had time to return to its true metabolic value the tolerance on O_2 should have been better than on air. In such a case a N_2 concentration effect would have obtained.

In Series 2, 3 and 4 the rate of ascent was increased so that unconsciousness was reached after 6, 3 and 1 minutes respectively. It was felt that some of these ascent rates should catch the maximum N_2 dilution effect. This one would expect to be maximal at the beginning of hypoxic ventilation. It can be seen that all 3 rates gave approximately the same difference in pO_2 level at which unconsciousness was produced. All these differences are highly significant as indicated by the standard error of the differences between the means as shown in the last column of Table I. The pO_2 difference can also be expressed in terms of altitude. In other words, if the N_2 dilution effect were not present in the air runs then the unconscious level would have been reached at an altitude of 28500 ft. (inspired pO_2 of 41) instead of 33000 ft. (inspired pO_2 of 31).

It should be pointed out that another factor might enter into the discrepancy of these two runs. That is the possible occurrence of aeroembolism in the oxygen runs which by itself may contribute to the lower pO_2 threshold. Furthermore, the criterion of the inspired pO_2 at B.T.P.S. ($T = 37°C$) is probably not strictly true at very low hypoxic levels when the metabolism and the body temperature sink rapidly in these small animals. This temperature drop would increase the pO_2 tension. However, this should affect both series and if effective at all would favor the 100% O_2 animals.

Summary. Mice were exposed progressively to equivalent degrees of hypoxia breathing air and breathing 100% O_2. The inspired pO_2 level at which unconsciousness set in was found to be significantly lower when air was breathed than when 100% oxygen was breathed provided the rate of ascent was 2×10^3 ft/min or greater. This advantage of air over oxygen is attributed to the transient "N_2 dilution" or R.Q. effect.

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Studies on the Localization of Tagged Methionine Within the Pancreas.*

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Recent studies of intermediary metabolism with isotopic compounds[1,2] have amply confirmed the concept of Schoenheimer[3] that tissue proteins are in a dynamic state of equilibrium with amino acids of exogenous source. It has been shown that different tissues show different activities in this respect,[4] and that intestinal mucosa is particularly active. Friedberg[5] postulated that the secretion of digestive enzymes and muco-proteins, which are rapidly lost from the tissue, would account for this rapid metabolic rate. On this basis he predicted that pancreas would show high activity, a fact actually shown earlier by Tarver and Schmidt.[6]

In balance studies, Gordon[7] showed that when labelled methionine, prepared with isotopic, radioactive sulphur (S^{35}), was given orally to animals, a high percentage of radioactive sulphur was recovered from pancreas, small intestine and liver. It was thought that such concentrations represent rapid incorporation of methionine into digestive enzymes; however, a possible role in the synthesis of hormones, particularly of insulin, cannot be excluded by ordinary balance studies. Attempts to demonstrate more accurate localization of isotopic compounds within tissues by radioautography have thus far been unsatisfactory in our hands. The present experiments attempt to localize the site of early methionine metabolism in the pancreas.

Experimental. Radioactive sulphur (S^{35}) was incorporated in methionine† starting with soluble sulphate. The specific activity of the final product (crystalline dl-methionine) approximated one microcurie per milligram (on date of receipt), based on a radioassay by another laboratory. In all cases this was administered intravenously in 2 ml water, and Table I shows the dosages given.

Preparation of and findings in the 4 cats in Table I were as follows:

No. 1 A normal cat. *Autopsy*: Pancreas was grossly normal; no sections were made.

No. 2 The pancreatic duct was ligated at the duodenum. Postoperative course was uneventful, and the animal gradually gained weight. Methionine was given 104 days after duct ligation. *Autopsy*: Grossly, there was complete late atrophy of the duodenal portion of the pancreas, partial atrophy of the tail of the pancreas (it is believed than an accessory duct preserved this portion). No sections were made.

No. 3 A ligature was placed around the body of the pancreas in its midportion. Postoperative course was uneventful, and weight was regained by the time of administration of labelled methionine 118 days after ligation. *Autopsy*: The pancreas appeared grossly normal proximal to the ligature, but there was apparent complete atrophy distal to the

* This investigation was conducted under the auspices of the Josiah Macy, Jr. Foundation, Conference on Liver Injury, and Sub-Committee on Tagged Methionine, with the participation of the Schools of Medicine of the Universities of Wisconsin, California, Pennsylvania, and Emory University.

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5 Friedberg, F., *Science*, 1947, **105**, 314.

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7 Gordon, E., personal communication.

† Grateful acknowledgment is made to Dr. Harry Fisher and the U. S. Industrial Chemical Company, Stamford, Conn., for synthesis of tagged methionine.

TABLE I.
Doses of Labelled Methionine Given and Content* of Radioactive Sulphur Found in the Pancreas.

Cat No.	Wt, kg	Methionine given, microcuries, per kg	Normal pancreas 4 hr	Normal pancreas 8 hr	Atrophic pancreas 8 hr
1	2.1	2.4	2970	3000	
2	2.9	1.7			1560†
3	3.1	2.4		2610	101
4	3.6	2.1		1500	8.1

* Standard specific activity ($\times 10^4$).
† From portion of pancreas which showed incomplete atrophy.

ligature. Sections showed normal pancreas proximal to the ligature; in the ligated portion, as seen in Fig. 1, there was patchy and incomplete atrophy of acinar tissue with occasional islets. The preservation of portions of acinar tissue is attributed to incomplete ligation.

No. 4 A ligature was placed around the body of the pancreas in its midportion. Again, postoperative course was uneventful, and weight was regained by the time of administration of labelled methionine 125 days after ligation. *Autopsy*: The pancreas appeared grossly normal proximal to the ligature, but there was apparent complete atrophy distal to the ligature. Sections showed normal pancreas proximal to the ligature. In the ligated portion, as seen in Fig. 2, there was marked atrophy of acinar tissue with intact islets, surrounded by fibrous tissue and lymphocytes.

All animals showed normal blood sugar concentrations after these operative procedures. In all cases methionine was given under light nembutal anesthesia. In Cats No. 1 and No. 2 pancreatic and liver biopsies were performed by laparotomy 4 hours after administration of methionine. All animals were sacrificed 8 hours after administration of methionine, and the organs and tissues were weighed, frozen and subsequently assayed.

The tissues were oxidized by digestion in Kjeldahl flasks containing Pirie reagent (perchloric acid-nitric acid-copper nitrate mixture), and soluble sulphate was precipitated with benzidine dihydrochloride. Sulphur content was determined by alkali titration, and sulphate was subsequently precipitated as barium sulphate. This precipitate was filtered with standardized apparatus on weighed paper, and radioactivity was directly measured with a thin-window, bell-type Geiger counter, the sensitivity of which was established by assay of a sample of known radioactivity. All dosages and counts for this laboratory are based on this standard. Assayed counts were corrected to a standard weight of barium sulphate, and were corrected for decay, based on the original assay. All results are based on duplicate analyses.

The radioactivity of samples, expressed as Standard Specific Activity (S.S.A.),[4] was calculated as follows, and is shown in Table II:

$$\text{S.S.A.} = \frac{100 \times \text{assayed counts} \times \text{weight of animal (kg)}}{\text{mEq sulphur in sample} \times \text{counts in dose}}$$

Discussion. It has been known since the work of Laguesse that the islet cells of the duct-ligated portion of a pancreas are physiologically intact, a fact which is supported in these animals by the morphological appearance of the pancreas. It would appear that the low concentration of radioactive sulphur found in tissue from the ligated portion, as compared with that from normal pancreas (Table I), is due to loss of acinar tissue. On this basis it appeared that the rapid early turnover of methionine by the pancreas is due to metabolic activity of the exocrine portion of the gland rather than to the needs of the islets. Some labelled methionine may be converted into cystine, which may then be incorporated in insulin by islet tissue, but, if so, this represents a small percentage of the unexpectedly high concentration of activity found in normal pancreas. The high concentrations found in liver, duodenum and jejunum (Table II) confirm the results of other investigators.

Summary. Methionine labelled with radioactive sulphur (S^{35}) was given intravenously to 4 cats, one normal, 3 treated so as to pro-

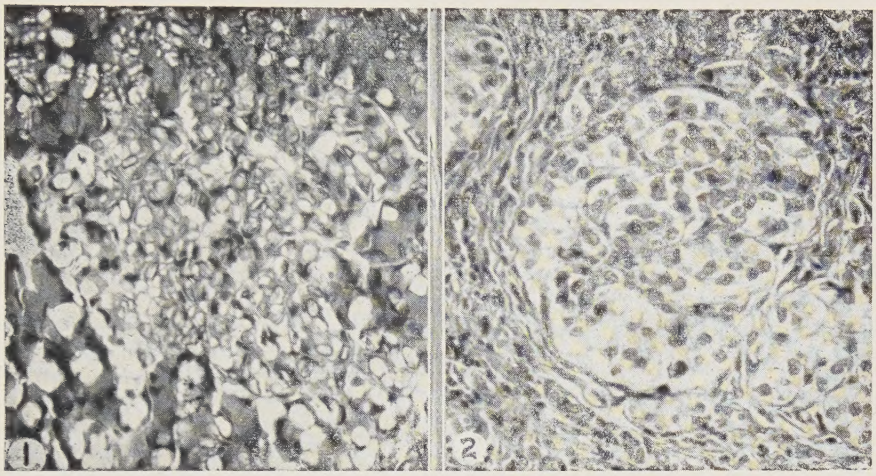

FIG. 1. Island of Cat 3. The cells of the island show some vacuolation and degranulation. Partial atrophy of the acinar tissue about the island is shown by the vacuolation and distortion of these cells.

FIG. 2. Island of Cat 4. The normal island is surrounded by fibrous tissue and lymphocytes. Atrophy of acinar tissue was complete in this portion of the animal's pancreas.

TABLE II.
Concentration of Radioactive Sulphur in Various Tissues.*

Cat No.	Duodenum	Jejunum	Liver	Bile	Kidney	Ovary	Testis
1	2185		1430		721		
2	1120		1540		684	77.1	
3	1440	882	1370		471	170	
4	840	672	848	528	699		16

* Expressed as standard specific activity ($\times 10^4$)

duce varying degrees of atrophy of the acinar tissue of the pancreas. The animals were sacrificed 8 hours after methionine was given, and various tissues were assayed for radioactive sulphur.

In all animals high activity was found in pancreas, small intestine and liver. In the atrophic portion of the pancreas where a ligature had been placed around the body of that gland, low activity was found as contrasted with high activity found in the normal part of the gland. Where atrophy was incomplete, the activity found was intermediate between that of normal and atrophic tissue.

It is concluded that the high activity of radioactive sulphur found in the pancreas of normal cats after administration of labelled methionine represents principally the metabolic activity of the exocrine portion of the pancreas.

(Continued from page iv)